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FRAXE-associated mental retardation protein (FMR2) is an RNA-binding protein with high affinity for G-quartet RNA forming structure

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ABSTRACT

FRAXE is a form of mild to moderate mental retardation due to the silencing of the *FMR2* gene. The cellular function of FMR2 protein is presently unknown. By analogy with its homologue AF4, FMR2 was supposed to have a role in transcriptional regulation, but robust evidences supporting this hypothesis are lacking. We observed that FMR2 co-localizes with the splicing factor SC35 in nuclear speckles, the nuclear regions where splicing factors are concentrated, assembled and modified. Similarly to what was reported for splicing factors, blocking splicing or transcription leads to the accumulation of FMR2 in enlarged, rounded speckles. FMR2 is also localized in the nucleolus when splicing is blocked. We show here that FMR2 is able to specifically bind the G-quartet-forming RNA structure with high affinity. Remarkably, *in vivo*, in the presence of FMR2, the ESE action of the G-quartet situated in mRNA of an alternatively spliced exon of a minigene or of the putative target *FMR1* appears reduced. Interestingly, *FMR1* is silenced in the fragile X syndrome, another form of mental retardation. All together, our findings strongly suggest that FMR2 is an RNA-binding protein, which might be involved in alternative splicing regulation through an interaction with G-quartet RNA structure.

INTRODUCTION

Fragile X E (FRAXE) mental retardation (OMIM 309548) is associated to a fragile site localized in Xq28 and is the cause of a non-syndromic X-linked mental retardation affecting 1/50 000 newborn males. The disorder is due to the silencing of the *Fragile Mental Retardation 2* (*FMR2*) gene, as a consequence of a CCG expansion located upstream to this gene. In the normal population, the number of this CCG repeat is variable between 6 and 35, while it is increased to more than 200 hypermethylated copies in FRAXE mentally retarded patients. The CCG repeat of FRAXE can either expand or contract and is equally unstable when transmitted through the male or the female germ line (1–3).

The FRAXE mental retardation is a form of mild to moderate mental retardation associated to learning difficulties, communication deficits, attention problems, hyperactivity and autistic behaviour (4). *Fmr2* inactivation generated mice displaying a delay-dependent conditioned fear impairment and a hippocampal increased long-term potentiation (LTP) (5). *FMR2* is a large gene with a major 8.75-kb transcript in placenta, fibroblasts adult and brain and a longer 13.7-kb FMR2 isoform in fetal brain (6). The *FMR2* gene is organized in 22 exons, showing several possibilities of alternative splicing for exons 2, 3, 5, 7 and 21. The longest of the *FMR2* isoforms is composed of 1272 amino acids and contains two nuclear localization signal (NLS) sequences that are both able to direct GFP into the nucleus (6). The nuclear localization of the endogenous FMR2 protein was also shown by

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immunohistochemistry in mouse brain (7). *FMR2* belongs to a gene family including *AF4* (8), *LAF4* (9) and *AF5q31* (10). These genes were originally cloned due to their fusion with MLL (mixed lineage leukaemia) in different chromosomal translocations causing acute lymphoblastic leukaemia (ALL). *AF4* is the best characterized member of this family, appears to play a role in transcription, since it interacts with Polymerase II (Pol II) and with the chromatin remodelling machinery (11). *AF4* deficient mice have an altered lymphoid development. Indeed in mouse, AF4 affects early events in lymphopoiesis, such as precursor proliferation or recruitment, but it is not required for the terminal stages of lymphocyte differentiation (12). The *FMR2* gene family has a common ancestor in *Drosophila*: the *Lilliputian* (*lilli*) gene, whose inactivation generates flies of reduced size. In addition, *lilli* mutant flies show reduced expression of some early zygotic genes such as *serendipity*, *fushi tarazu* and *huckebein*, that are essential for cellularization and embryonic patterning (13).

By analogy with AF4, FMR2 has been considered to be a putative transcription activator. However, it is important to underline that the ability of FMR2 to activate transcription was proven only for some domains, but not for the full-length protein (14). The purpose of this study was to unravel the function of FMR2 that is currently unknown. We show here that FMR2 is localized in SC35-containing nuclear speckles, being implicated in splicing. This role in splicing is carried out through its specific interaction with G-quartet RNA, a structure known to be able to act as an exonic splicing enhancer (ESE) (15). We also show here that FMR2 influences *in vivo* the alternative splicing pattern of the mRNA of *Fragile X Mental Retardation 1* (*FMR1*), which contains a G-quartet nearby an acceptor site of the alternatively spliced exon 14 (16).

MATERIALS AND METHODS

Plasmids constructions

Full-length *FMR2* cDNA was amplified by RT–PCR from NG108 RNA using LA Taq (TaKaRa) and primers whose sequences are reported in Table 1 containing the XhoI and BamHI restriction sites. PCR fragments were digested with restriction enzymes and cloned into the Flag-pTL1 plasmid (17). Three regions of *FMR2* (N-ter, C-ter, C1) were amplified from full-length gene and subcloned into Flag-pTL1 vector (17) using the same restriction enzymes. The full-length *FMR2*, N-ter and C-ter domains cloned in Flag-pTL1 were amplified by PCR using the primers whose sequences are reported in Table 2 and cloned in a pET-151D Topo plasmid (Invitrogen).

The FBS and FBSΔ35 fragments were amplified from pTL1-N19 and pTL1-N19 Δ35 plasmids, respectively (18) using the following primers: 5′-GGGTCGACGAAGAG AGGGAGAGCTTC-3′ (forward) and 5′-GGGGATCCG TTTCCTTTGAAGCCTCCTC-3′ (reverse). The primers contain the SalI and BamHI restriction sites, respectively. The PCR fragments generated using these primers were digested and subcloned either in the SXN 13 minigene (19) or in the pGEM-T easy vector (Promega). All plasmids were verified by sequencing.

Table 1. Primers used to clone full-length *FMR2* and its deletion constructs in Flag-pTL1

Constructs	Forward and reverse primers
Full-length	5′-GGCTCGAGGATCTATTCGACTTTTTCAG-3′
N-ter	5′-GGGGGATCCCTACAACAAGTGGGCATCG-3′
	5′-GGCTCGAGGATCTATTCGACTTTTTCAG-3′
	5′-GGGGATCCGGCTGGTTTGTGGGCAGTGGC-3′
C-ter	5′-GGCTCGAGGCCACTGCCCACAAACCAGCC-3′
	5′-GGGGGATCCCTACAACAAGTGGGCATCG-3′
C1	5′-GGCTCGAGGCCACTGCCCACAAACCAGCC-3′
	5′-GGGGGATCCCAGTGGTGGTGACAATGGC-3′

Table 2. Primers used to clone *FMR2* and its deletion constructs in pet-151D Topo

Constructs	Forward and reverse primers
FMR2	5′- CACCGATCTATTCGACTTTTTCAG-3′
	5′-CTACAACAAGTGGGCATCGATG-3′
N-ter	5′- CACCGATCTATTCGACTTTTTCAG-3′
	5′-CTAGGCTGGTTTGTGGGCAG-3′
C-ter	5′-CACCGCCACTGCCCACAAACCAG-3′
	5′-CTACAACAAGTGGGCATCGATG-3′

Antibodies, immunofluorescence and immunoblot

To generate the polyclonal anti-FMR2 antibody, a synthetic peptide—CPEQKNRMIPSHQETTHSS—corresponding to amino acids 116–133 of mouse FMR2 was coupled to ovalbumin and used for immunization of rabbits by standard protocols (Eurogentec). The characterization of this antibody is described in Supplementary Material. The antiserum was affinity purified as described previously (20).

Transfection and immunofluorescence were carried out as previously described (20) using the following antibodies: polyclonal anti-FMR2 at 1:1000 (this study), monoclonal anti-Flag M2 (Sigma) at 1:1000, polyclonal anti-Flag (Sigma) at 1:1000, monoclonal anti-SC35 (Abcam) at 1:2000, and the following secondary antibodies: Alexa fluor 488 anti-rabbit IgG or Alexa fluor 594 goat anti-mouse IgG (Molecular Probes). Immunoblot was carried out as described (20) using polyoclonal anti-FMR2 (1:1000) (this study), monoclonal anti-Flag M2 (1:3000) (Sigma).

Cell culture

HeLa and NG108 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. Human fibroblasts were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin.

Actinomycin D treatment, microinjection

NG108 and HeLa cells were incubated with Actinomycin D (ActD) (Sigma) at the final concentration of 5 μg/ml.

Cytoplasmic microinjection of an antisense U6 oligonucleotide in SK-N-SH cells was performed as described (21) using lysine-fixable Cascade blue-conjugated dextran (Invitrogen) as a marker to identify injected cells. Cells were fixed 2 h after injection or 2–4 h after ActD treatment.

Production and purification of recombinant proteins

The FMR2 full length, N-ter and C-ter regions of FMR2 were amplified by PCR from its cDNA using the primers listed in Table 2. After purification, the PCR fragments were cloned in a pET-151D Topo plasmid (Invitrogen) as fusion proteins with a six histidine tag. The clones were expressed in *Escherichia coli* BL21 star (DE3) (Invitrogen). The cells were grown in LB medium with ampicillin (100 μg/ml), induced for 4 h by addition of 1 mM isopropyl-β-D-thiogalacto-pyranoside (IPTG) when the cultures reached an OD of 0.4 at 600 nm. The cells were harvested and resuspended in lysis buffer [25 mM Tris–HCl pH 7.6, 300 mM KCl, 1 mM EDTA, 1 mM DTT, 20% glycerol, 5% NP-40, 0.5 M urea, complete protease inhibitor cocktail (Roche), 1 mM PMSF] sonicated for 5 min and centrifuged at 15 000 r.p.m. for 30 min at 4°C. The supernatant was incubated with Ni-NTA Agarose (QIAGEN) overnight at 4°C by agitation. Beads were washed four times with washing buffer (25 mM Tris–HCl, pH 7.6, 300 mM KCl, 1 mM DTT, 0.5 M urea, 20% glycerol, 20 mM imidazole). Fusion proteins were eluted from the beads with elution buffer (25 mM Tris–HCl pH 7.6, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1% Triton, 0.5 M urea, 200 mM imidazole) for 30 min at 4°C.

RNA binding assays

RNA homopolymer binding assay were performed as described (20). Filter binding assay and gel retardation assay were performed as previously described (18,22).

Reverse transcriptase–polymerase chain reaction

Semiquantitative PCR was performed to evaluate the mRNA produced by minigene constructs, using the primers SXN13 alternative exon reported in Table 3.

We evaluated the inclusion of exon 2 of the SXN13 minigene and of exon 14 of *FMR1* gene by quantitative RT–PCR as we have recently described (23). The sequences of primers and PCR conditions used in this set of experiments are reported in Table 3. RNA was purified from HeLa cells, normal and FRAXE fibroblasts using the RNeasy kit (Qiagen) and retro-transcribed by the Thermoscript RT-PCR System (Invitrogen). Real-time PCR was performed using a 7000 Sequence Detection System (Applied Biosystem) using the cDNA, qPCR Core kit for SYBR Green (Eurogentec) according to the manufacturer's instructions and 200 nM of each primer. All experiments were performed in triplicate. The relative expression of transcripts was quantified using $2^{-\triangle\triangle CT}$ method (24). Human *GAPDH* was used as endogenous control.

PCR amplification for real-time PCR was performed with 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR Green to double-stranded DNA.

Table 3. Primers used to quantify relative expression of minigene SXN13 and *FMR1* mRNA

Gene	Forward and reverse primers
SXN13 alternative exon	5′-GACCATTCACCACATTGGTG-3′ 5′-GAACCTCTGGGTCCAAGG-3′
Primers junction SXN13	5′-GCCCTGGGCAGGTCGAC-3′ 5′-GACCACCAGCAGCCTGGA-3′
FMR1 alternative exon	5′-GGAACAAAGGACAGCATCGC-3′ 5′-CCAATCTGTCGCAACTGCTC-3′
FMR1 constituve exon	5′-CATGCACTTTCGGAGTCTG-3′ 5′-GAAATCTCGAGGCAAGCTG-3′
GAPDH	5′-CCACATCGCTCAGACACCAT-3′ 5′-GACCAGGCGCCCAAT-3′

RESULTS

Localization and functional domains of FMR2

To get insight into FMR2 function, we performed a detailed study of its intracellular localization by generating a polyclonal anti-FMR2 antibody using a synthetic peptide corresponding to amino acids 116–133 of mouse FMR2 (see Supplementary Material). We then transfected HeLa cells (which do not express endogenous FMR2, Figure1 and Supplementary Figure 1) with a construct encoding full-length FMR2 and we revealed its nuclear localization in a dot-like pattern (Figure 1A). This same result was obtained with a Flag-tagged-FMR2 (not shown). Our anti-FMR2 antibody recognizes endogenous FMR2 protein in neuroblastoma NG108 cells (Figure 1B) and in primary hippocampal neurons (Figure 1C). Intriguingly, both endogenous and transfected FMR2 are present in the nucleus in large dots colocalized with nuclear speckle domains, as revealed by the anti-SC35 monoclonal antibody (Figure 1, Supplementary Figure S2). These nuclear domains represent sites where splicing factors are concentrated, assembled and modified (25) (Supplementary Figure 2).

To define the functional properties of FMR2 domains, we divided FMR2 into three regions and subcloned them fused to Flag-tag: N-ter (residues 1–640), C-ter (residues 633–1272), C1 (residues 633–966). We used these constructs to transfect HeLa cells and their protein products were revealed by immunofluorescence using the anti-Flag antibody. The N-ter construct is localized in the cytoplasm (Figure 2), while C-ter exhibits the same nuclear distribution as the full-length protein, co-localizing with SC35 and C1 is nucleolar (Figure 2) (in Supplementary Figure 2B the co-localization of C1 with fibrillarin as a nucleolar marker is shown). We conclude that the C-ter domain is determinant for the speckle localization of FMR2. Interestingly, this domain also contains a nucleolar localization signal in its C1 subdomain.

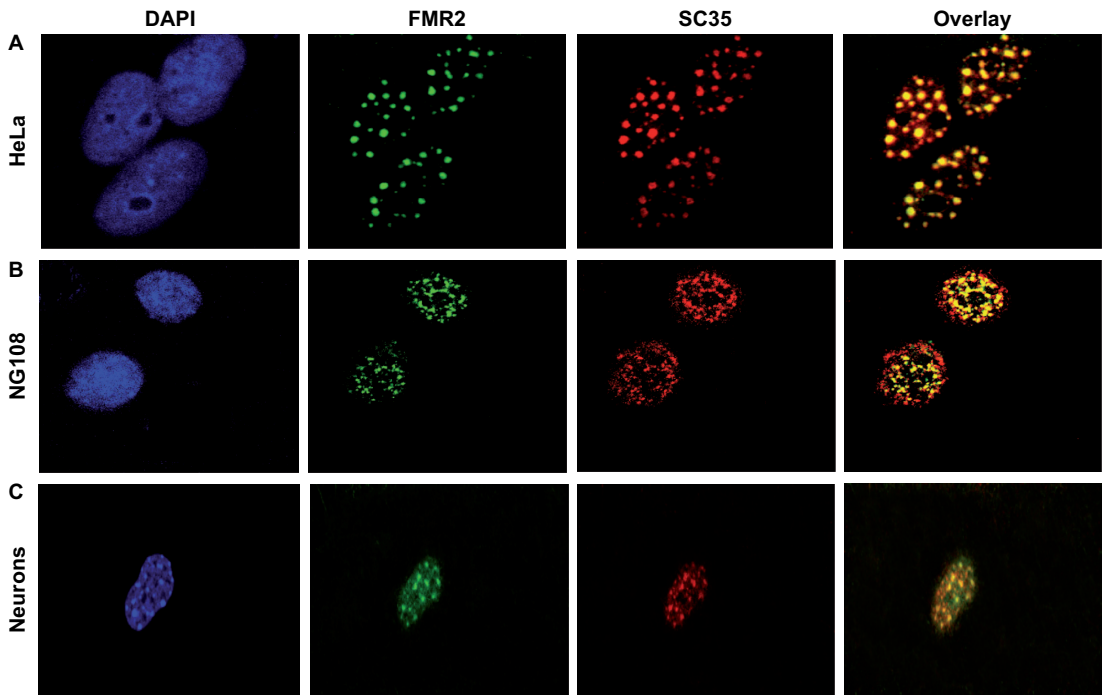

Figure 1. Endogenous and overexpressed FMR2 localizes to nuclear speckles. Co-localization of FMR2 with SC35 in nuclear speckles as detected by polyclonal anti-FMR2 antibody in Hela cells transfected with full-length *FMR2* (**A**) and in NG108 cells (**B**) and in primary hippocampal neurons (**C**) expressing endogenous FMR2. FMR2 was detected with polyclonal anti-FMR2 antibody and SC35 was detected by monoclonal anti-SC35 antibody. (A) 63× magnification, scale bar 5 μm. (B and C) 40× magnification, scale bar 10 μm. Twenty-five 40× fields were analyzed, showing a comparable result.

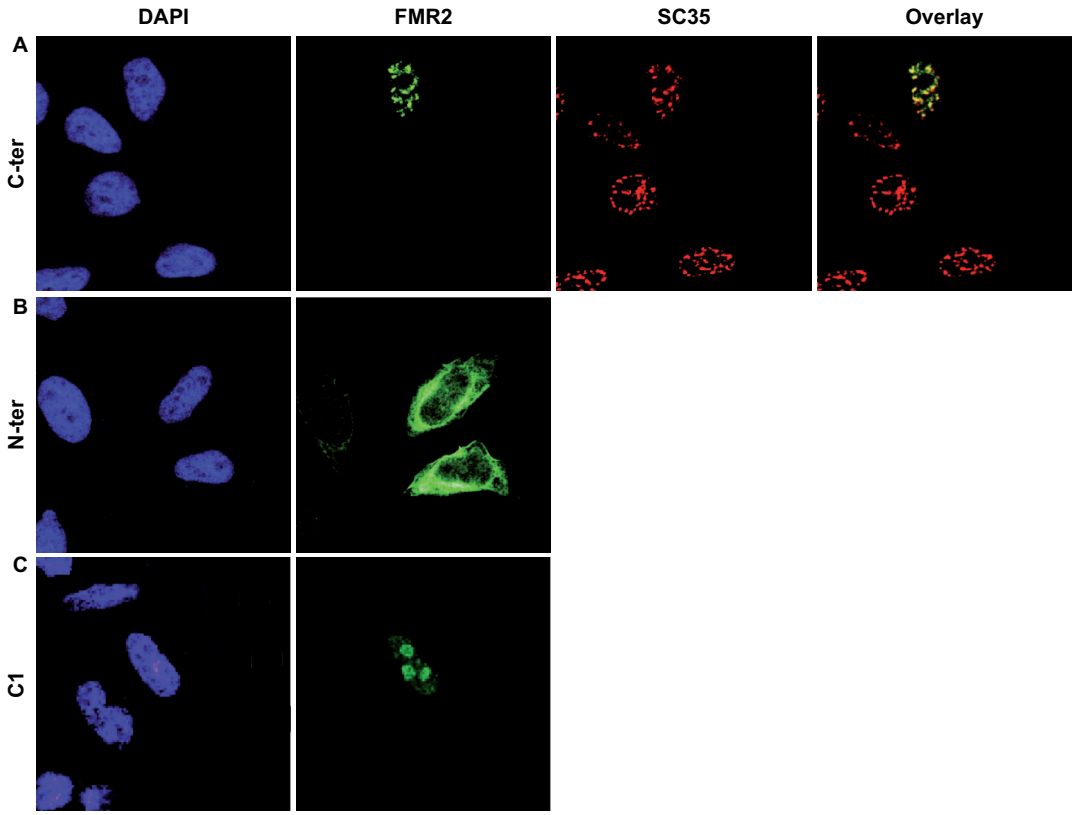

Figure 2. FMR2 determinants for its intracellular localization. (**A**) Co-localization of C-ter with SC35 in nuclear speckles in Hela cells. (**B**) Localization of N-ter in the cytoplasm of HeLa cells. (**C**) Nucleolar localization of C1 in HeLa. All FMR2 domains have been detected using the polyclonal anti-Flag antibody, and SC35 was detected by the anti SC35 monoclonal antibody. The 40× magnification, scale bar 10 μm. Twenty 40× fields were analyzed, showing a comparable result.

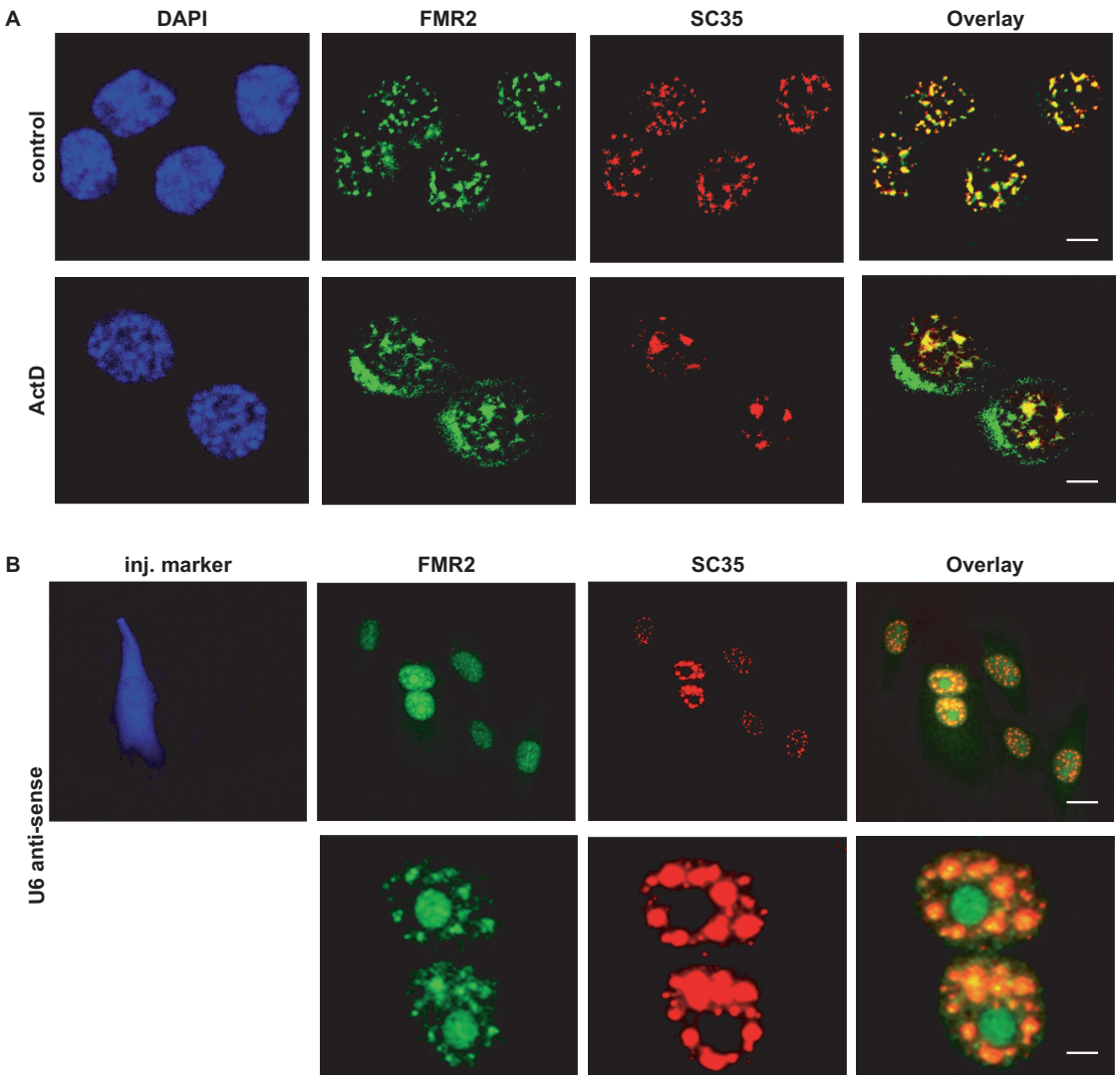

Figure 3. Blocking of transcription and splicing affects nuclear speckles localization of FMR2. (**A**) Blocking of transcription in NG108 neuroblastoma cells by ActD. The treatment affects the morphology and number of nuclear speckles, as revealed by detection of SC35 with anti-SC35 antibody. FMR2 is co-localized with SC35 in control cells and after treatment, as detected by the anti-FMR2 antibody. In ActD-treated cells, FMR2 is also localized in the cytoplasm. The 40× magnification, scale bar 10 µm. Twenty-five 40× fields were analysed, showing a comparable results. (**B**) Blocking of splicing in SK-N-SH neuroblastoma cells. In U6 antisense-microinjected cells, SC35 is accumulated in enlarged nuclear speckles, as detected by monoclonal anti-SC35 antibody. In these cells, FMR2 is co-localized with SC35 and is also concentrated in the nucleoli, as detected by polyclonal anti-FMR2 antibody. Upper panels, 20× magnification, scale bar 10 µm; lower panels, 63× magnification, scale bar 5 µm. Ten 20× fields were analysed, showing a comparable result.

Dynamic behaviour of FMR2

Nuclear speckles are dynamic structures whose size and shape vary according to the level of RNA polymerase II-dependent transcription and splicing activity (25). For this reason, we investigated whether inhibition of splicing or transcription have an impact on FMR2 intracellular localization (21,26). First, we studied the subcellular distribution of SC35 and FMR2 in NG108 cells after blocking transcription by ActD. As expected, SC35 appears redistributed into enlarged, rounded speckles (Figure 3A), similar to other splicing factors (26). Interestingly, we observed that a portion of FMR2 protein is co-localized with SC35. We also blocked splicing in living cells by injecting the antisense U6 oligonucleotide in the SK-N-SH neuroblastoma cell line. This led to the accumulation of SC35 in enlarged speckle domains (Figure 3B). In these conditions FMR2 is partially co-localized with SC35 in these speckles-like structures and also concentrated in nucleoli (Figure 3B). Taken together, these data strongly suggest that FMR2 is involved in splicing and may have RNA-binding properties.

FMR2 is an RNA-binding protein

To validate our hypothesis, we tested the ability of FMR2 to bind synthetic RNA homopolymers. Indeed, FMR2 selectively binds to poly (G), but not poly (A), poly (C) or poly (U) (Figure 4A) while the control FMRP (Fragile X Mental Retardation Protein), as expected, is able to bind to poly (G) and poly (U) (20). The binding activity to poly (G) was retained by the FMR2 C-ter domain, the

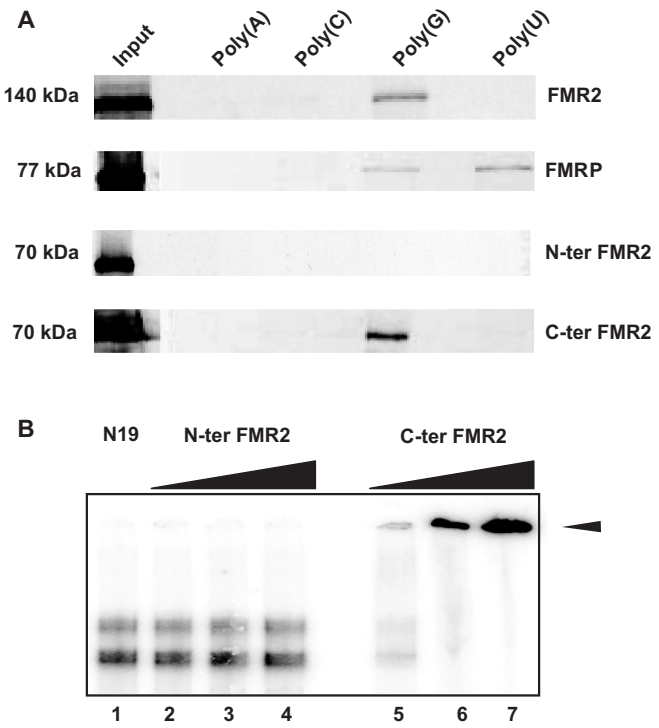

Figure 4. FMR2 is an RNA-binding protein.(**A**) *In vitro* translated [^{35}S] methionine labelled full-length FMR2, full-length FMRP, N-ter and C-ter proteins were incubated with each RNA homopolymer linked to agarose in the presence of 0.25 M KCl. The same volume (10 µl) of each eluate was analysed by SDS–PAGE followed by fluorography. (**B**) Labelled N19 probe was incubated in the presence of increasing amounts of recombinant N-ter (lanes 2–4: 0.2, 0.4 and 0.6 pmol, respectively) and C-ter (lanes 5–7: 0.2, 0.4 and 0.6 pmol, respectively) proteins. As a control, the labelled N19 probe was shown in lane 1.

same region directing its localization in nuclear speckles (Figure 2), but not by the N-ter domain (Figure 2). Even if no homology with other RNA-binding domains is present in FMR2, two putative RNA-binding motifs were identified within this region (the first between residues 787 and 815 and the second between residues 890 and 917) using the RNABindR program (27). Since FMR2 has affinity for poly (G) RNA, we questioned whether it can bind any RNA containing a stretch of G nucleotides or if it is able to recognize and bind a specific G-rich RNA structure. For this reason, we tested the ability of N-ter and C-ter recombinant FMR2 proteins to bind a RNA probe containing a G-quartet forming structure, the N-19 RNA in a gel retardation assay. Previously, we have shown that *FMR1* mRNA contains a G-quartet forming structure, the N19 sequence, localized between nucleotides 1470 and 1896 of *FMR1*, which is bound by FMRP itself (18,28). Indeed, as for the poly G-sequence increasing amounts of C-ter bind the N19 probe while of N-ter does not interact with the RNA (Figure 4B), suggesting a specificity of FMR2 for G-quartet structure via its C-terminal domain.

FMR2 has high affinity for the G-quartet RNA structure

G-quartets or quadruplexes are four stranded nucleic acid structures formed by stacking of planar layers of guanine tetrad units. In the tetrads, four guanines interact two by two in a cyclic Hoogsteen hydrogen bonding arrangement. G-quartet RNA folds in a 3D structure preferentially in the presence of K+ ions in comparison with Li+ and Na+ ions (18,29), as it was also shown for G-quartet RNA structure localized in *FMR1* mRNA (18,22). To test our hypothesis, we generated recombinant N-ter, C-ter and full-length His-tagged FMR2 proteins and we performed a filter-binding assay with the N19 probe, using FMRP as a positive control. Surprisingly, in the presence of K+, full-length FMR2 and C-ter proteins were able to bind N19 as tightly as FMRP, whereas the N-terminal domain, in agreement with the results shown in Figure 4A and 4B, was not (Figure 5B). Additionally, in the presence of Na+, that partially destabilizes the G-quartet formation, FMR2 and C-ter bound to N19 to a much lower extent (Figure 5C), while in the presence of Li^+ the binding of FMR2 proteins to N19 RNA was abolished (Figure 5D). To measure binding affinity we used competition assays that are more sensitive and allow for alleviation of the contribution of the non-specific binding properties of FMR2 (18,22). Therefore a constant concentration of labeled N19 RNA was incubated with a fixed amount of His-FMR2 and C-ter in the presence of increasing concentrations of unlabeled N19 RNA as a competitor. Binding of FMR2 and C-ter to the labeled N19 RNA was efficiently competed by the cold probe (Figure 5E). As little as 1 nM of competitor RNA is able to displace 50% of FMR2 from the G-quartet labeled probe, an affinity comparable to that of FMRP for G-quartet RNA structure (17,18,22). When we used the unlabelled N8 competitor RNA (corresponding to the 3′UTR of *PP2Ac* mRNA and not containing G-quartet RNA) (17) as a negative control, no displacement of the binding was observed for FMR2 (Figure 5E). Then we tested the ability of FMR2 and C-ter to bind the FBS (FMRP binding site) RNA, encompassing only the G-quartet forming structure inside the N19 RNA (Figure 5A) (nt 1557–1658 of the *FMR1* mRNA coding region), FMRP, FMR2 and C-ter proteins were able to bind specifically to FBS (Figure 5F). Conversely, when we deleted the G-quartet structure generating the ΦBΣ Δ35 sequence [described in Figure 5A and in ref. (18)] full-length FMR2 and its C-terminal domain were unable to bind (Figure 5G). We can conclude that FMR2 binds G-quartet RNA specifically and with high affinity.

FMR2/G-quartet RNA interaction *in vivo*

The G-quartet being a purine-rich element, it has features of an exonic splicing enhancer (ESE), a group of discrete sequences within exons that promote both constitutive and regulated splicing (30). In particular, the G-quartet present in the *FMR1* mRNA has been recently shown to be a potent ESE (15). To confirm these data, we introduced the FBS sequence into the middle exon of the SXN13 minigene (19), generating SXN/FBS and we transfected HeLa cells with one or the other minigene. The presence of a G-quartet determines the complete inclusion of exon 2 in the SXN/FBS mRNA, which was detected by semiquantitative RT–PCR using primers localized in exon

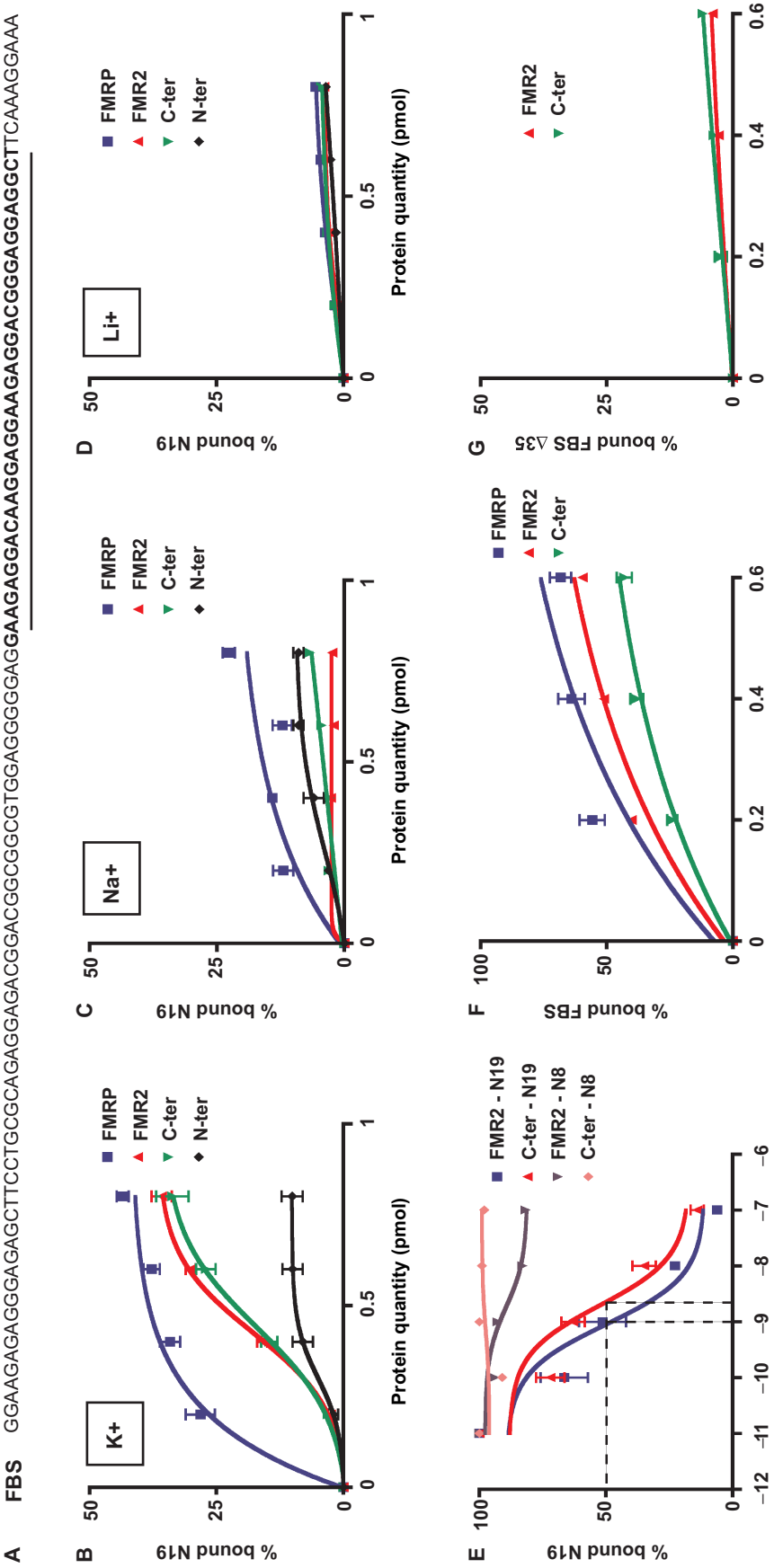

Figure 5. FMR2 binds with high specificity the G-quartet RNA structure. (**A**) Sequence of the FBS purine-rich region encompassing the G-quartet forming structure inside the N19 RNA. The 35 nucleotides indicated in bold and underlined were deleted to generate the ΦBΣ Δ35 sequence. (**B**) Filter-binding assay using increasing amounts of full-length FMR2, FMRP, N-ter and C-ter proteins in the presence of K^+ using the N19 RNA as labelled probe. (**C**) The same experience described in (**B**) was repeated in the presence of Na^+ and in (**D**) in the presence of Li+. (**E**) Competition experiments in a nitrocellulose binding assay using the N19 unlabelled RNA as competitor and the unlabelled 3′UTR of *PP2Ac* RNA (N8) not containing any G-quartet forming structure as a negative control. (**F**) Filter-binding assay using an increasing amount of full-length FMR2, FMRP and C-ter proteins. The labelled probe is FBS RNA. (**G**) Filter-binding assay using an increasing amount of full-length FMR2 and C-ter proteins. The labelled probe is the ΦBΣ Δ35 RNA. Each point shows the mean of the results obtained in three independent experiments (see Supplementary Table 1 for details of each binding assay).

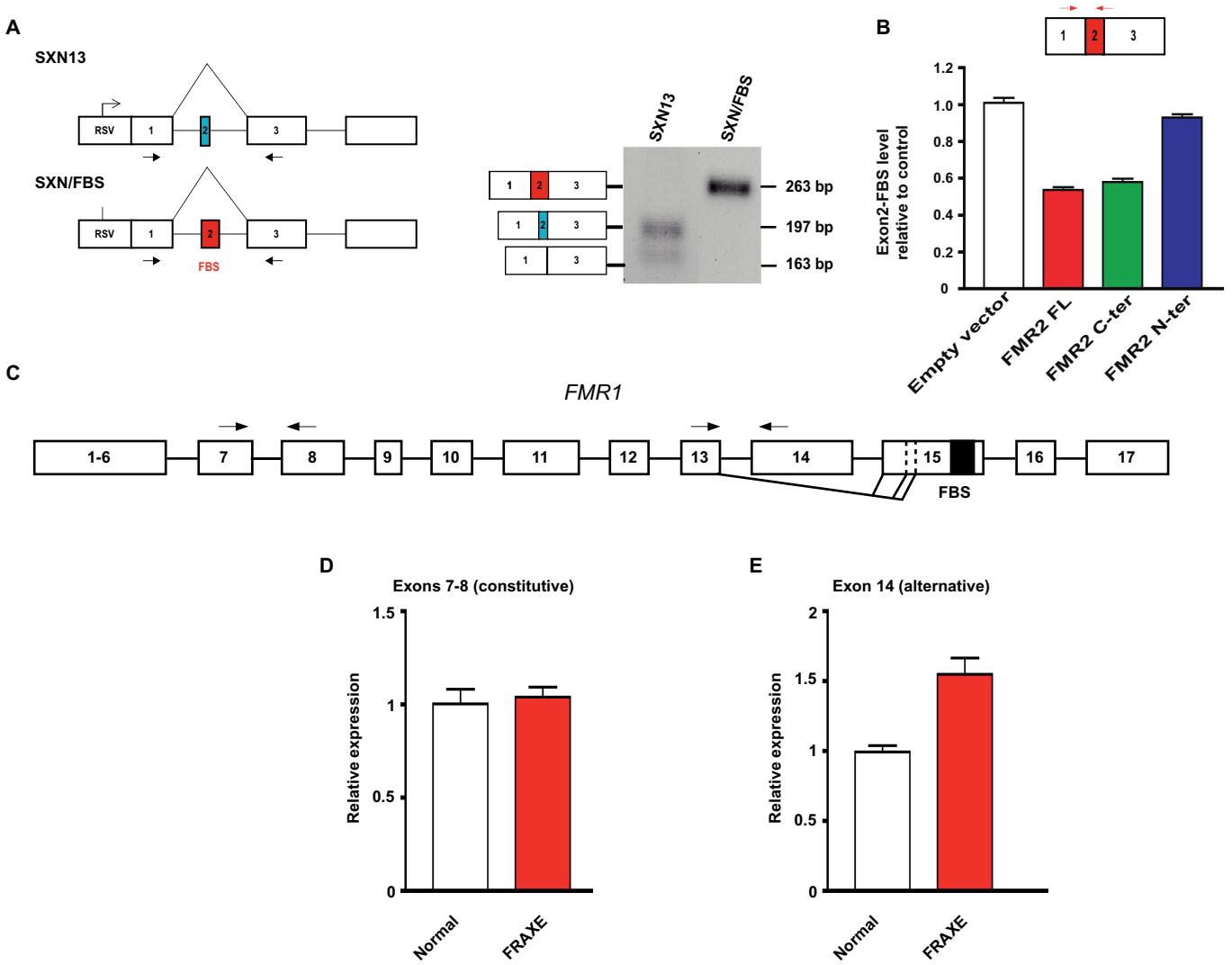

Figure 6. Effect of FMR2 on splicing of an alternative exon in minigene-transfected cells and in the endogenous *FMR1* transcript. (**A**) Schematic representation of SXN13 minigene and of SXN13/FBS minigene. This last one includes G-quartet in exon 2. Visualization of splicing products of the minigene by semiquantitative RT–PCR. Black arrows indicate primers positions. (**B**) Relative expression of the inclusion of minigene exon 2 using real-time quantitative PCR in the presence or in the absence of FMR2, C-ter and N-ter proteins. Results were obtained by analysis of three independent transfection experiments. Red arrows indicate primers postitions, *GAPDH* was used as a standard. Error bars represent standard deviation. (**C**) Schematic structure of the *FMR1* full-length mRNA, showing alternative splicing of exon 14. (**D**) Relative expression of *FMR1* constitutive exons 7–8 using real-time quantitative PCR in normal fibroblasts and in fibroblasts obtained from a patient carrying the deletion of *FMR2* gene. No difference is observed between the two samples. (**E**) Relative expression of exon 14 sequence-including *FMR1* isoforms using real-time quantitative PCR in normal fibroblasts and in fibroblasts obtained from a patient carrying the deletion of *FMR2* gene. An increased expression level of 50% is observed for exon 14-containing *FMR1* isoforms mRNA in FRAXE fibroblasts.

1 and 3 of the minigene (Figure 6A). Starting from this observation, we tested whether FBS/FMR2 interaction could regulate the splicing of the SXN/FBS minigene. To this purpose, we co-transfected HeLa cells with the SXN/FBS minigene and with full-length FMR2, C-ter, N-ter or the empty vector. Since differences in the expression of the SXN/FBS minigene were difficult to appreciate in non-quantitative conditions, we evaluated the relative expression of transcripts with alternative exon 2 inclusion by real-time quantitative PCR (primers are localized in exon 1 and exon junction between exon 2 and 3 of minigene. Their sequences are described in Table 3). As shown in Figure 6B, inclusion of the exon containing FBS was decreased about 2-fold in cells co-transfected with full-length FMR2 or C-ter relative to cells co-transfected with the empty vector or with the N-ter. In this experiment, the expression level of each FMR2 protein was tested by western blot and quantitative RT–PCR, as illustrated in Supplementary Figure 3. To exclude that this result is due to a different decay of the mRNA coding for minigene isoforms in the presence or in the absence of FMR2, we tested the stability of SXN/FBS minigene in HeLa cells transfected and treated with ActD for 6 h. No difference in decay of exon2-containing minigene mRNA was observed during this treatment in cell transfected with FMR2-pTL or the pTL empty vector (Supplementary

Figure 4A). Also in this case, the expression level of FMR2 was tested by western blot (Supplementary Figure 4B) using the monoclonal anti-Flag antibody. This finding suggests that the FBS/FMR2 interaction modulates and influences the splicing efficiency of a minigene containing FBS and that this function is retained by the C-ter, but not by the N-ter domain, which does not bind to the FBS. These data suggest that inclusion of a G-quartet-containing exon in a mature mRNA might be modulated *in vivo* by FMR2. We asked whether the absence of FMR2 has an impact on the alternative splicing pattern of the *FMR1* gene, containing a G-quartet structure in its exon 15 near to the 3′ acceptor sites necessary for alternative splicing of exon 14 (16). This structure is a potent exonic splicing enhancer [(15) and this study]. For this reason, we tested the expression level of exon 14-containing *FMR1* isoforms in normal fibroblasts and in a cell line of a FRAXE patient carrying a deletion of *FMR2*, that completely abolishes the expression of this gene (1). For this quantification, we used real-time PCR using different sets of primers: a couple of primers amplifying the N-terminal region of *FMR1* mRNA (exons 7 and 8) and a couple of primers located in exon 13 and exon 14 (Figure 6C), giving the possibility to evaluate the inclusion of the exon 14 sequence in the *FMR1* mature mRNA. Interestingly, the overall expression of *FMR1* (all isoforms) is not influenced by the absence of *FMR2* (Figure 6D), while in FRAXE fibroblasts the inclusion of exon 14 in *FMR1* mRNA is increased of 50% when compared with normal fibroblasts (Figure 6E). This demonstrated altered inclusion of exon 14 in the mature mRNA of *FMR1* in the absence of FMR2.

DISCUSSION

FMR2 expression is silenced in FRAXE mental retardation and its precise function is not known. Previous studies have been suggested that FMR2 function is related to transcriptional regulation in the nucleus. This assumption was based on the observation that FMR2 was exclusively detected in the nuclear compartment and that short N-terminal fragments of the protein have been reported to act as transcriptional activators of the adenovirus E1b minimal promoter (14). This function was also supported by analogy and homology with AF4, a member of the same gene family, that has been reported to interact with PolII transcription elongation factor b (P-TEFb) kinase and in complex with AF9, ENL and AF10 mediates methylation of histone H3-K79 (11). Our study reveals an additional important function of FMR2, providing a novel insight on the molecular basis of FRAXE mental retardation by providing evidence that FMR2 is an RNA binding protein having a putative role in the regulation of splicing of the G-quartet structure-containing mRNA. We show here that FMR2 has high affinity for RNA and, in particular, we demonstrate its specificity towards G-quartet RNA. G-quartet RNA motifs are known to play a role in translational control (17,31) and in controlling splicing efficiency working as an ESE [(15) and this study]. We show that FMR2 is co-localized with SC35 in the nuclear speckle domains, the compartments where splicing factors are stored, assembled and modified (25). Interestingly, blocking pre-mRNA splicing induces the redistribution of a subpopulation of FMR2 molecules in nuclear speckles containing the SC35 splicing factor, while another pool of the protein accumulates in the nucleolus. This observation suggests that FMR2 shuttles between speckles and splicing sites, where it can pick up cargo RNAs and transfer them to the nucleolus where they can undergo subsequent modifications. Indeed, the nucleolus performs additional roles beyond generating ribosomal subunits, since about 5% of nucleolar components are proteins involved in mRNA processing (32). Intriguingly, the ability of the herpes virus saimiri ORF57 protein to export of virus mRNA to the cytoplasm is dependent on its nucleolar localization, suggesting a critical role of the nucleolus in mRNA export process (33). On the other hand, blocking transcription by ActD treatment determines FMR2 localization within enlarged nuclear speckles-like structure and also its accumulation in the cytoplasm. Indeed, it is known that inhibition of PolII transcription by actinomycin D induces the accumulation of some RNA-binding proteins and transcription factors in the cytoplasm (e.g. hnRNP A1 protein) where they are confined when no exported RNA is synthetized in nucleus (34). Taken together, our data suggest a possible role of FMR2 in post-transcriptional modifications of RNA and, in particular, in splicing. We validated this hypothesis using a model minigene system which allowed us to demonstrate that the over-expression of FMR2 has an impact on the inclusion of the alternative exon 2 containing the G-quartet structure bound by FMR2 in the mature RNA. Indeed, we show here that the presence of FMR2 does not influence the stability of the minigene mRNA. Moreover, we tested the expression level of exon-14 containing *FMR1* isoforms in fibroblasts null for *FMR2*. The interest to study these splicing variants is due to the fact that *FMR1* exon 14 encodes a nuclear export signal (NES) sequence. Exon 14-containing *FMR1* isoforms display cytoplasmic localization, while exon 14-lacking isoforms are localized in the nucleus (16,35). Interestingly, the absence of FMR2 does not affect the total expression of *FMR1* mRNA but increases the expression of the isoforms containing exon 14 and encoding a protein localized in the cytoplasm.

The role of FMR2 in splicing is not exclusive to its role in transcriptional regulation, since we cannot exclude that the function of FMR2 is modulated by the interaction with transcription factors, as, shown, for example, for SWI/SNF, a complex involved both in chromatin remodelling and regulation of alternative splicing (36). Remarkably, the N-terminal region of FMR2 is able to affect transcription (14), while we show here that its C-terminal domain modulates splicing. Furthermore, one of the proteins belonging to the same family as FMR2, AF4, has been shown to stimulate RNA polymerase II transcriptional elongation and mediate coordinated chromatin remodeling (11). We can also add that the role of transciption factor in post-transcription RNA metabolism is more and more evident, as, for example, recently reported (37). Indeed, these authors have shown the

molecular mechanism by which RA/RARα regulates translation of specific mRNA in neuronal dendrites (37). Even the orphan nuclear receptor DAX-1 was shown to have a role in the mRNA metabolism (38).

Our data indicate a role of FMR2 in splicing of *FMR*1 pre-mRNA. This suggests that alteration of FMRP expression in FRAXE patients may participate in the physiopathology of this syndrome. The partial effect of FMR2 on *FMR1* exon14 inclusion may be due to partial rescue of FMR2 function by its homologous proteins, which are expressed in a broad range of tissues, as also recently described for RBM5 and its homologues RBM6 and RBM10 (39). Alternatively, it is worth to underline that probably other RNA-binding proteins control the complex splicing pattern of *FMR1* with a different impact in different tissues, as shown for NMDA receptor 1 (NR1). The inclusion of exon 19 into mature NR1 mRNA is in fact regulated by the balanced action of several RNA-binding proteins: hnRNP A1, hnRNP H, Nova and NAPOR (40).

A long list of RNAs exists containing putative G-quartet structures that are located in the proximity of an alternatively spliced site (http://bioinformatics.ramapo.edu/grsdb/) (41). Subtle alterations of the alternative splicing of some of these genes (e.g. *FMR1, ATRX*, *Neuroligin 3*, etc.) in the absence of FMR2 may then be relevant to the pathogenesis of the FRAXE syndrome. Indeed, mutations in ATRX cause a form of mental retardation (42) and *Neuroligin 3* has been shown to be implicated in autism (43), a phenotype also described in FRAXE patients (4).

In conclusion, here we have described a novel function FMR2 in splicing. While further analysis will be necessary to completely identify all the FMR2 targets mRNAs and its precise mechanism of action, our data show that FMR2 is a multifunctional protein linking RNA metabolism and transcriptional control.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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