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ABSTRACT

The fragile X mental retardation protein (FMRP) is a RNA-binding protein proposed to post-transcriptionally regulate the expression of genes important for neuronal development and synaptic plasticity. We previously demonstrated that FMRP binds to its own FMR1 mRNA via a guanine-quartet (G-quartet) RNA motif. However, the functional effect of this binding on FMR1 expression was not established. In this work, we characterized the FMRP binding site (FBS) within the FMR1 mRNA by a site directed mutagenesis approach and we investigated its importance for FMR1 expression. We show that the FBS in the FMR1 mRNA adopts two alternative G-quartet structures to which FMRP can equally bind. While FMRP binding to mRNAs is generally proposed to induce translational regulation, we found that mutations in the FMR1 mRNA suppressing binding to FMRP do not affect its translation in cellular models. We show instead that the FBS is a potent exonic splicing enhancer in a minigene system. Furthermore, FMR1 alternative splicing is affected by the intracellular level of FMRP. These data suggest that the G-quartet motif present in the FMR1 mRNA can act as a control element of its alternative splicing in a negative autoregulatory loop.

INTRODUCTION

The most frequent cause of inherited mental retardation, fragile X syndrome, is caused by the absence of the RNA-binding protein Fragile X Mental Retardation (FMRP). In neurons, FMRP is associated with a limited subset of brain mRNAs together with other proteins within large ribonucleoparticles, the composition of which is still incompletely known (1–3). Within these mRNPs, FMRP is proposed to act as a regulator of translation or transport of specific target mRNAs. However, the molecular mechanisms of FMRP action on specific target mRNAs are still poorly understood. As a clue to the function of FMRP, the study of its mRNA targets appears an essential step. The guanine-quartet (G-quartet) structural motif was identified as a high affinity determinant of the interaction of FMRP with mRNAs (4,5). RNA G-quartet is not the only proposed target of FMRP since U-rich sequences (6), a kissing-loop motif (7) and the BC-1 RNA (9) were also found to mediate the interaction of FMRP with mRNAs. However, FMRP target mRNAs bearing the kissing-loop motif have not yet been identified and the interaction mediated via BC1 is under debate (8).

To address these questions, we analyzed in this work the interaction between FMRP and its own mRNA, FMR1, one of the best characterized targets of FMRP where the G-quartet motif had been identified (2). Because the

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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interaction between FMRP and its own mRNA was suggestive of an autoregulatory loop, we tested whether FMRP could control its own expression. To determine the function of the \textit{FMR1} mRNA/FMRP interaction, we performed mutations within the G-quartet motif of the FMRP binding site (FBS) of \textit{FMR1}, which abolished FMRP binding \textit{in vitro} without changing the amino acid sequence of the protein and we examined at which level the FMRP/FBS interaction could play a role. Our data provide several lines of evidence for a role of the FBS and its binding to FMRP in alternative splicing regulation of the \textit{FMR1} gene.

**MATERIALS AND METHODS**

**Plasmids and constructions**

Plasmid pTLL1 (18) was used to transiently or stably express \textit{FMR1} longest isoform 1 in the various cell lines described in text. Flag and cMyc tags were introduced in frame at N-terminus of \textit{FMR1} to give pTLL1-Flag-\textit{FMR1} and pTLL1 cMyc-\textit{FMR1}. Mutations disrupting G-quartet within the FBS were introduced into pTLL1-Flag-\textit{FMR1} using Quick Change Site Directed Mutagenesis kit (Stratagene, Cedar Creek, TX, USA). Primers used for mutagenesis are given in Supplementary Material available online. The SXN13 minigene constructions (19) were produced by inserting dsDNA fragments of FBS within exon 2 using \textit{SalI/BamHI} sites. Plasmid pTAP–\textit{FMRP} was constructed by inserting \textit{FMR1} Iso1 in frame at its N-terminus with TAP tag of pBS 1539 (20) into \textit{MluI} site of pTRE2 vector (Clontech, Mountain View, CA, USA).

**Cell culture and transfections**

HeLa cells and \textit{FMR1}−/− mouse embryonic immortalized fibroblasts (21) were cultured in DMEM supplemented with 10% fetal bovine serum, 100 \mu g/ml penicillin-streptomycin. PC12 Tet-On cells (Clontech) were grown in RPMI supplemented with 10% horse serum, 5% fetal bovine serum, 125 \mu g/ml hygromycin, 100 \mu g/ml of penicillin-streptomycin, in a 5% CO₂ incubator at 37 °C. PC12 Tet-On cells were stably transfected with pTAP–\textit{FMRP} using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer recommendations. The pHyg resistance vector was used in the cotransfection as a selection marker. Transfected cells were cultured in medium containing 125 \mu g/ml hygromycin and 1 \mu g/ml doxycyclin, and individual double stable selected cells were tested for the presence of the TAP–\textit{FMRP} fusion protein by western blot using 1C3 anti-FMRP. PC12 Tet-On clone ‘1’ was selected for its tight regulation of TAP-\textit{FMRP} expression. To induce exogenous human FMRP Iso1 expression in stably transfected cell lines, doxycyclin was added to cells to a final concentration of 250 ng/ml for 48 h.

For the determination of SXN minigene splicing efficiency, HeLa cells or \textit{FMR1}−/− mouse embryonic immortalized fibroblasts at 40% confluency were transfected with 1.5 \mu g SXN vector using JetPEI (Polyplus) in 60 mm diameter plates. After 24 h, total RNA was extracted using Genelute mammalian total RNA kit (Sigma, Steinheim, Germany) and 5 \mu g was used for extension with the SXN primer described below.

**Primer extension**

Primer extensions to detect G-quartet structure within RNAs were performed as described in (4) using primer 5′-TCCATCTGTGGTCTCCTT for \textit{FMR1} and 5′-AGAACCTCTGGGTCAAGG for SXN minigene Exon 2.

**RNA-binding assays**

RNA-binding assays were performed using RNAs T7 \textit{in vitro} transcribed labeled with [\alpha-\textit{32}P]ATP. Affinities were determined using competition gel shift assays with GST–\textit{FMRP} as described previously (4). Briefly, \textit{32}P-labeled \textit{FMR1} mRNA fragment N19 encompassing the FBS was incubated with 0.1 pmol GST–\textit{FMRP} in the presence of increasing concentrations of unlabeled N19 or mutant N19-\textit{ΔG4} competitor RNA.

**Polysomes preparation**

Polysomes were prepared from four 10 cm diameter confluent HeLa cell plates. Twenty minutes before harvest, 90 \mu g/ml cycloheximide was added to cultures. Cells were lysed in 200 mM Tris–HCl pH 7.5, 5 mM MgCl₂, 100 mM KCl, 10 U/ml RNasin (Promega, France), 1 mM DTE, 0.5% NP40 at 4 °C. Supernatant of 10 min centrifugation at 13 000 r.p.m. was loaded onto 15–45% sucrose gradient run 2 h at 36 000 r.p.m. at 4 °C. Polysomal fractions were precipitated with 0.1 M NaCl and 2.5 vol. ethanol and the mRNAs from these fractions were purified with GenElute Mammalian Total RNA kit (Sigma).

**\textit{In situ} hybridization**

\textit{In situ} hybridization were performed as described in (22) using oligonucleotide modified with fluorophore CY3 (GE Healthcare, France) and directed against the Flag sequence of \textit{FMR1} (5′-CTTGTCATCGTCG TCCCTGATGCCATGAAATTCGCCCTATA).

**Western and northern blots**

Immunoblot analyses were performed with IC3 antibody (1/2000), anti-Flag (1/1000 Sigma), anti-cMyc (1/500, Ozyme, France) and anti-β-actin (1/1000) as described (18).

Northerns were performed according to (23). Radioactive probes were prepared using kit ‘dsDNA all-in-one-random-prime’ (Sigma) with \textit{FMR1}-3′ UTR and 28S rRNA encoding cDNAs.

**Real-time PCR**

Total RNA (1 \mu g) prepared with Genelute mammalian total RNA kit (Sigma) was retrotranscribed with Superscript III (Invitrogen) using random priming, and real-time PCR were performed using the Brilliant SYBR-Green QPCR Core Reagent Kit (Stratagene) on MX4000 apparatus (Stratagene). The following oligonucleotides were used for qRT–PCR.16Ra 5′-GTGGACGATTATC TGTTCCGGGAA, R15/16 5′-CGTCTTTCCTTTGAAA GCC, P14/15F 5′-GATATACTTCAGGAACTAATTC, 5′-CGTCTTTCCTTTGAAA GCC, P14/15F
p14/15.1F 5'-GATATACTTCAGCTCCAACAG, p14/15.2F 5'-GATATACTTCAGCTCCAACAG, 11/13F 5'-CAAAAGTCAGAGGGGGAGT, 5'UTR-FMR1.R 5'-GGCAAGGAGGACGGAGAAGAT, 5'UTR-FMR1.R 5'-TTGTGGAATCTCATCATGG, R13/15.1 5'-CA GAATTAGTCTTTAATAGTAG, R13/15.2 5'-CTTGGTGGAGCTTTAATAGTAG, F-GAPDH 5'-GGATGCGAGGGAGTAGTGGTC and R-GAPDH 5'-TGCACCAACCACTGCG.

2D-PAGE
Protein extraction and first dimension: cells were harvested by centrifugation and resuspended in 10 mM Tris, 1 mM EDTA and 250 mM sucrose. Lysis was performed with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% ampholytes, 20 mM DTT). DNA was eliminated by 3 min sonication. A total of 100 μg of proteins were diluted in 135 μl of rehydration buffer, which were used to rehydrate Biorad ReadyStrip IPG pH 3–10 strips. Isoelectric focusing 30 min at 500 V and 250 Vh, 30 min at 1000 V and 500 Vh and 1 h at 4000 V and 8000 V using the MultiphorII system (GE Healthcare). Second dimension: strips were equilibrated for 20 min in 50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 50 mM DTT. Strips were placed on vertical 1.0 mm 10% SDS polyacrylamide gels and sealed with 0.5% agarose sealing solution. Electrophoresis was performed in standard running buffer at 150 V for 1 h.

RT–PCR
Total RNA was prepared from cortices of 10 days old wild-type (Wt) or FMRP−/− male mice using Trizol reagent (Invitrogen) followed by RNeasy purification (Qiagen, Hilden, Germany) and their synaptoneurosomal fractions were prepared according to (24). Total RNA (1 μg) was retrotranscribed with Superscript® (Invitrogen) using random priming. One microliter of RT reaction (1/10) was used to perform PCR reactions in 25 μl reaction volume with the following primers: F13 5'-GTGGGAAACAAAAAGACGATCG, R15 5'-CCT CTGGCGCAGGAGCTC, R4 CACCAACAGCAAAG GCTCTTT, F2-3 5'-TTGAAAAACACTGGCAAACCA, F-GAPDH and R-GAPDH. Reactions were performed as follows, initial denaturation 3 min at 95°C, then 30 s at 95°C, 30 s at 60°C and 30 s at 72°C, with 40 cycles.

RESULTS
The FBS contains two independent G-quartet structures stabilized by adenesines
FMRP specifically binds to its own mRNA both in vitro (4,25,26) and in cells (27). The binding site of FMRP on its mRNA, here called the FBS, consists of a G-quartet motif present in the C-terminal coding region of FMR1 mRNA (4). The G-quartet motif is formed by the stacking of several guanine tetrat units. Adenesines were also suspected to contribute to the structure in the FBS by forming intercalating adenine quartets. To investigate the function of the interaction between FMRP and its own mRNA, we constructed a series of mutants to inhibit FMRP/FBS interaction by disrupting the G-quartet structures. Previous work had suggested the presence of two distinct G-quartet structures (4). To test this hypothesis, two sets of mutations were constructed to disrupt either one or both potential structures, called ΔG1 and ΔG2 (Figure 1A). In a first step, mutations were essentially substitutions of As to Cs and Us at the wobble position of codons to preserve the encoded FMRP protein sequence and to test the contribution of adenesines. The presence of G-quartets in the RNAs was indicated by the presence of potassium-dependent stops of reverse transcription as previously described (4). ΔG1 mutation, located around position 1613, suppressed the 1613 (G1) stop while the stop at position 1647 (G2) remained unchanged (Figure 1B). Conversely, ΔG2 mutations had the opposite effect, with the 1613 stop unchanged and the stop 1647 suppressed. These results indicate that two independent G-quartet structures exist in the FBS. Moreover, because the mutations left the guanine content of the FBS essentially unchanged while substituting several adenesines, our results support a role for the adenesines in stabilizing both FBS G-quartets. This stabilization effect can be explained by the formation of A-tetrads stacking within the G-quartet structure as previously proposed (4). When both sets of mutations were combined in mutant ΔG(1+2), the stop at position 1647 reappeared while the stop at position 1613 remained absent (Figure 1B). The reappearance of a G-quartet structure at G2 position within the ΔG(1+2) RNA despite the presence of mutations ΔG2 could be explained by the fact that the double mutant generated a different and more stable G-quartet structure because the G-content was essentially not affected by the mutations.

FMRP binding to its own mRNA has no impact on FMR1 translation
We tested next the ability of these different mutant RNAs to interact with FMRP by gel shift assay as previously described (4). The mutant RNAs (ΔG1 and ΔG2) bound to FMRP with the same affinity as for the Wt FBS (data not shown). This indicated that FMRP equally binds one or the other structure. To completely disrupt G-quartet formation within FBS, a new set of mutations, consisting essentially of A to C substitutions at the wobble position of codons and favoring hairpin structures, was performed (Figure 2A). The mutations were inserted in full length FMR1 mRNA and the disruption of the G-quartet structure was confirmed by reverse transcriptase (RT) elongation test (Figure 2B). We have shown previously that a 425-long RNA fragment (N19) of FMR1 mRNA containing the FBS recapitulated a Wt-binding efficiency (4). To confirm the loss of interaction of FMRP on ΔG4-FBS, the mutations were inserted also in the N19 fragment (N19-ΔG4) and its interaction with FMRP was tested using gel shift assay (Figure 2C). The binding efficiency of N19-ΔG4 RNA was found to be decreased by more than a 100-fold compared with Wt N19 RNA (Figure 2D). This level of interaction,
in the micromolar range, was assigned to nonspecific binding as previously determined (4).

The impact of the disruption of G-quartet structures within the FBS was then analyzed in various cell types [HeLa, Cos-7, and fibroblasts from FMR1/C0/C0 mice (21)] by transiently or stably expressing FMR1 bearing/C1G4 mutation. In these cells no difference in FMRP protein level could be detected between the cells expressing Wt or/C1G4 FMR1 (Figure 3A). Also, no difference could be detected between Wt and mutant FMR1 mRNA levels (Figure 3B). Furthermore, although mRNAs bearing G-quartets had been reported to be differently associated with polyribosomes in the absence of FMRP (10), we could not detect a change in the association of/C1G4-FMR1 mRNA with polysomes both in HeLa and in FMR1/C0/C0 mouse fibroblasts (Figure 3C). Finally, we did not observe any significant difference between Wt and/C4 FMR1 mRNAs localization in HeLa cells (Figure 3D). Thus, we concluded that the interaction between FMRP and the FBS had no detectable impact on FMR1 mRNA stability, translation and localization in the tested cells.

The FBS is a potent exonic splicing enhancer

A number of facts brought us to examine next a potential implication of the FBS in splicing. Firstly, the FBS is located nearby to alternatively spliced sites of FMR1 (Figure 4A). Secondly, because of its high purine content, the sequence of the FBS has analogies to an ESE consensus (28). Third, because of its shuttling activity, FMRP has been proposed to bind mRNAs already in the nucleus and therefore should be able to interact with pre-mRNAs (29,30).

The ability of the FBS to act as an ESE in vivo was tested by using the SXN13 minigene system (19) derived from the β-globin gene and composed of four exons, one of which (exon 2) being alternatively spliced (Figure 4A). The presence in exon 2 of a sequence with ESE properties (12MU3) induced exon 2 inclusion and resulted in a longer mRNA product (Figure 4C and E). A fragment of the FBS still able to form a G-quartet structure or its corresponding/C1G4 mutant was inserted within the second exon of the minigene to determine its ESE properties (Figure 4C and D). After transient transfection in HeLa cells of the plasmids bearing the different
minigene constructions, RT elongation was directly performed on the total RNA extracted from the cells using a 5' end 32P-labeled oligodeoxynucleotide priming within exon 3 of the minigene. The ESE properties of the FBS fragment were evaluated by measuring the ratio between the long RT product (bearing exon 2) and the short RT product (without exon 2) of the alternative splicing of the globin minigene. While the 12MU3 sequence was capable to specify exon 2 inclusion in about 80% of the splicing events (Figure 4E), the G4-FBS fragment induced a complete inclusion of the exon 2. Meanwhile, exon 2 was totally excised in the G4-FBS mutant. These data indicated that the FBS had potent exonic splicing enhancing properties on a minigene and these properties were linked to its ability to form a G-quartet structure.

The overexpression of one FMRP isoform alters FMR1 alternative splicing pattern in PC12 cells

The fact that the FBS had a potent ESE activity in a minigene suggested that FMRP could regulate its own splicing by binding to FBS. To verify this hypothesis, we first tested whether the splicing efficiency of a globin minigene bearing the FBS fragment could be influenced by FMRP. Splicing of SXN13-G4-FBS minigene was analyzed in FMR1/C0/C0 mouse fibroblasts (21). In these cells, the expression of either FMRP major cytoplasmic isoform 7 or nuclear isoform 6 (18) by transient or stable transfection, had no detectable influence on SXN13-G4-FBS expression (data not shown). An absence of effect of FMRP on the minigene system could be due to the fact that the FBS was out of its natural context or had a too strong ESE effect on minigene splicing.

We then tested the influence of an overexpression of FMRP on FMR1 pre-mRNA splicing. The FBS is located close to two alternative splice sites within exon 15 of FMR1. The 3' side of the FBS is located 110, 74 or 35 nucleotides downstream of the three different acceptor 5' ends of exon 15 (4,31). The alternative splicing occurring at these three sites leads to six types of exon 15 variants, depending whether exon 14 is skipped or not. These three acceptor sites are used in different proportions in various...
Figure 3. FMRP binding on its own mRNA has no effect on FMR1 translational regulation. (A) Western blot analysis of pTL1 Flag-FMR1 and pTL1 Flag-ΔG4-FMR1 expression in HeLa cells. HeLa cells (6 × 10⁶ cells) were transfected with the indicated amount plasmids (µg). Westerns blot on 15µg total cell extracts using anti-cMyc, anti-Flag and anti-ßactin antibodies, revealed no difference between Wt and mutant ΔG4-FMR1 encoded protein levels (one of three independent experiments is presented, P < 0.05, similar results were obtained in Cos-7 and in FMR1−/−fibroblasts). (B) Northern blot analysis of FMR1 mRNA expression level with 15µg of HeLa cell total RNA extracts using probes specific of pTL1 encoded FMR1 mRNAs and of 28S rRNA as internal control. The pBS is control lane without FMR1 encoded plasmid. No difference is observed between Wt and ΔG4 expression levels. (C) Localization of Wt and mutant ΔG4 FMR1 mRNAs in polyribosomes of HeLa cells. In the upper part is depicted a typical profile of polyribosomes separated on a 15-45% linear sucrose gradient registered at 254 nm optical density. The lower graphic represents the quantification by qRT–PCR of the FMR1 mRNA in the indicated pooled fractions using GAPDH mRNA as internal control. No significant difference was observed between Wt and ΔG4-FMR1 mRNAs in their localization in the different ribosome subsets. Similar results were obtained in the FMR1−/−cells. (D) Intra-cellular localization of Wt and ΔG4 mRNAs by fluorescence in situ hybridization in HeLa cells. Cy3 labeled anti-Flag oligo-deoxynucleotide probe (Flag) revealed a similar cytoplasmic and perinuclear localization for both mRNAs. DAPI staining of the nuclei is shown.

tissues (32) suggesting the possibility of regulation at this level. Because the FBS is highly conserved (4) we tested the possible impact of FMRP/FBS interaction in rat cells. Rat pheochromocytoma PC12 cells stably transfected with a tagged human isoform 1 FMRP (iso-1h) under the control of the inducible promoter Tet-On were used (PC12-1′ cells). We tested in these cells, the effect of iso-1h FMRP increase on endogenous (rat) FMR1 mRNA alternate splicing. Upon induction of iso-1h expression (Figure 5A) by doxycyclin treatment of the cells, the total amount of FMR1 mRNAs (rat + human) was found increased over 30-fold compared to its basal level in PC12 cells (Figure 5B left). Meanwhile, the global level of endogenous FMR1 mRNAs (rat) was not affected (Figure 5B right). The splicing events taking place around the FBS site within the endogenous FMR1 were analyzed by qRT–PCR using rat specific primer sets (Figure 6A). Our data showed that the products of exon 15 first acceptor site usage (including the longest isoform 1, the most frequent isoform 7 and isoforms 13 and 17) were decreased over 2-fold (Figure 6B). This decrease was concomitant with an increase in minor isoforms, products of exon 15 second and third acceptor site usage (1.4- and 1.8-fold respectively), including the minor isoforms 2, 3, 8, 9, 14, 15, 18 and 19. Thus, the overexpression of the full-length FMRP isoform alters FMR1 splicing events around the FBS in a manner that indicates a displacement of the equilibrium between major and minor isoforms. These data are in agreement with the hypothesis that FMRP binding to the FBS plays a role in regulating FMR1 splicing.

We examined also the splicing events leading to exon 14 skipping. Upon overexpression of the full-length FMRP, all transcripts lacking exon 14 were found decreased by 2-fold. Although the splicing events leading to exon 14 skipping are likely in relationship with those occurring between exons 14 and 15, they are quite rare events compared with the latter ([26] and our data not shown). To confirm the alterations of FMR1 expression seen at the RNA level upon iso-1h overexpression, we analyzed FMRP isoform expression by western blotting after 2D PAGE. The use of monoclonal anti-FMRP IC3 antibody (recognizing an N-terminal epitope) indeed revealed significant differences in FMRP isoforms upon iso-1h overexpression (Figure 6C). The identification of each protein spot is however extremely difficult due to the complexity of splice products. The highest product visible only in iso-1h expressing cells (Figure 6C, right, spot a) could correspond to the exogenous iso-1h transferred inefficiently due to its higher molecular weight. Spots b and d, which showed a broadening in the PC12-1′ cells compared to PC12, likely contained several isoform species of similar molecular weight. The origin of spot c, which appeared in PC12-1′ cells was unknown. Most remarkable is the decrease of spot f, which could correspond to the shortest FMRP isoforms 10 and 11 (48 and 47 kDa respectively) that were found decreased at mRNA level. Altogether, these data showed that an overexpression of one FMRP isoform was able to alter FMR1 alternative splicing pattern both at RNA and protein level.
The splicing pattern of *FMR1* exon 15 is altered in the cortex of *FMR1*−/− mice

Following the observation that an overexpression of FMRP in cells leads to an alteration of its splicing at exon 15, we tested next whether the absence of FMRP could lead to similar defects. Thus, we analyzed the splicing pattern of *FMR1* mRNAs in Wt and *FMR1*−/− mice where FMRP protein is absent but *FMR1* mRNA is still expressed. *FMR1*−/− mice have been produced by the insertion of a neomycin cassette within exon 5 (33). The splicing events were analyzed by RT–PCR (Figure 7A) on total RNA extracted from cortices of 10-days old Wt and *FMR1*−/− mice as well as in the synaptosomal fractions (SN) of these extracts where FMRP function is considered to be prominent (34). In parallel, RT-PCRs were also performed on another part of *FMR1* mRNA (exon 3, Figure 7D) and on GAPDH mRNA (Figure 7E) for normalization. As previously reported, while FMRP protein expression was abolished in *FMR1*−/− (Figure 7C), *FMR1* mRNA remained expressed, although reduced to about 65% of Wt level (Figure 7A and 7D, compare lanes 3 to 4 and 5 to 6), possibly due to NMD events. RT–PCR performed across exon 15 (Figure 7A) revealed three bands corresponding to the three isoforms produced by the alternative branching of exon 14 and exon 15. Not surprisingly the isoforms lacking exon 14 are not detected in these PCR conditions because they are much less frequent events (26). Comparison of Wt and *FMR1*−/− exon 15 RT-PCR products (Figure 7B) revealed a marked difference concerning...
the smaller products that correspond to minor spliced mRNAs variants. Thus, these exon 15 minor splices disappeared in FMR1<sup>−/−</sup>, both in total cortical extracts and in SN fractions (Figure 7A lanes 4 and 6). These data indicate that FMRP absence alters the splicing pattern of FMR1 exon 15 in the cortex. While an overexpression of FMRP led to an increase of exon 15 minor splices, the absence of FMRP had the opposite effect. Altogether these data support a role for FMRP in the control of its own splicing at exon 15.

**DISCUSSION**

In this work, we analyzed the functional impact of the interaction between FMRP, the protein absent in the fragile X syndrome and the binding site identified in its own mRNA (4). We previously demonstrated that the FBS is located within the region encoding the RGG domain of FMRP. One main structural feature of this site is its ability to adopt a guanine quadruplex or G-quartet motif. We showed here that the structure of the FBS was more complex than initially thought. Thus, we identified two independent G-quartet structures in the FBS. Mutations that abolished either one or the other structure (mutant ΔG1 and ΔG2) had no impact on FMRP binding efficiency in the context of a 425-nt long fragment (N19), indicating that FMRP can indistinctly bind to either one or the other structure. Furthermore, we showed that several adenines of the FBS play a role in the differential stability of the G-quartet structures, supporting the initial hypothesis that the structure involves intercalating adenine quartets (4) and as already observed for other G-quartet structures (35). Substitution of these adenines by pyrimidines does not however prevent formation of a G-quartet structure within the FBS and does not affect binding to FMRP in vitro. The elimination of both structures (mutant ΔG4), while keeping the encoded protein sequence unchanged, dramatically reduced FMRP binding to a non specific level and confirmed the absolute requirement for a G-quartet for efficient binding. We then tested the impact of mutation ΔG4 within the context of full FMR1 mRNA in cells. Surprisingly, no effect of G-quartet absence could be detected neither on mRNA translation and localization nor on polyribosomes association in HeLa cells. Thus, these observations do not support a role in a translationally controlled autoregulatory loop of the binding of FMRP to its own mRNA as initially proposed (4). The fact that the FBS site is purine-rich and localizes close to alternative splicing sites was suggestive of its potential function as a splicing regulator of FMR1. Indeed, mammalian ESEs were identified initially as purine-rich sequences that associate with specific SR-family proteins and promote the utilization of adjacent splice sites (28). When a fragment of the FBS that retained its ability to form a G-quartet was tested in a minigene system, a strong exonic splicing enhancer activity was observed. This activity was completely abolished in a mutant that had lost its ability to form the G-quartet although it kept a G-rich sequence (ΔG4). Thus, our data indicate that the FBS is a potent ESE and interestingly, the ESE activity of FBS seems to rely on its ability to adopt a G-quartet structure. These data suggest that the FBS may be a control element of FMR1 alternative splicing and the binding of FMRP could play a role in the control. Indeed, we showed that the equilibrium between short and long FMRP isoforms produced by exon 15 alternative splicing is altered by manipulating the level of FMRP protein (either by overexpression of the longest isoform 1 or in FMR1 KO cells where the FMR1 mRNA is still expressed). This supports the idea that FMRP binding to the FBS site controls the ratio between the different isoforms in an autoregulatory loop. The binding of FMRP longest isoform 1 on the FBS could counteract or modulate its ESE function (for instance by interfering with SR proteins) such as to favor the minor site inclusion. The two alternative G-quartet structures are equidistant (39 and 36 nt respectively) from the two alternative splicing sites in exon 15, suggesting that they could act as a molecular switch for controlling exon 15 alternative splicing. However, one cannot exclude at present that the observed effect of FMRP on the alternative splicing of its own mRNA may be indirect, involving for instance the translational control by FMRP of splicing factors.

The biological significance of a modulation of FMR1 alternative splicing is presently unclear in particular because it is not known whether the different isoforms of FMRP, some of which being present in very low amount, have different functions. Still a variation in their ratio is likely to have implication for the function.
of FMRP. For instance, the isoforms lacking the 5' end of exon 15 produced by the alternative splicing at second and third acceptor sites both lack serine 499, the major known phosphorylation site of FMRP (36,37). This phosphorylation site was shown to modulate FMRP association to mRNAs in drosophila (37) and to affect translation in mammalian cells (36). Based on our observations, increased FMRP binding to FBS would result in a decrease in the synthesis of FMRP major isoforms (carrying a complete exon 15) together with an increase of minor

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**Figure 6.** The overexpression of exogenous FMRI isoform 1 within PC12 cells alters FMRI splicing pattern. (A) Schematic structure of FMRI region subjected to alternative splicing with the list of rat specific sets of primers used for the analysis. The primer localization with the isoforms they enable to measure is given. (B) Quantification by qRT–PCR of different FMRI isoforms ratio between PC12 (white bars) and PC12-l' (black bars) cells using the primer sets presented in (A). Data are means from qRT–PCR triplicates, normalized with GAPDH and using at least two independent RNA preparations. Values from PC12 were arbitrarily set to 1. *Student test \( P < 0.05. \) (C) Western blot with anti-FMRP (1C3) antibodies on PC12 and PC12-l' cell extracts separated on 2D PAGE. Spots described in the article are identified from a to f. pH ranges are indicated at the top of the 2D PAGE.
isofoms (lacking serine 499) downregulating FMRP function in a negative autoregulatory loop.

In conclusion, while we could not show a translational effect of FMRP binding to its own FMR1 mRNA, our data support the implication of the FMRP/G-quartet interaction on the regulation of FMRP alternative splicing around exon 15. The fact that perturbations of the intracellular level of FMRP leads to modulation of exon 15 isoforms expression in a way susceptible to alter their RNA-binding properties suggests the existence of a possible autoregulatory loop. Our data suggest also that FMRP might be involved in splicing regulation of other genes containing G-quartet motifs in their protein coding sequence. This should be particularly prominent in neurons where FMRP is expressed at its highest level and even locally in dendrites where FMRP is present and splicing has been proposed to occur (38).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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3. Garber,K., Smith,K.T., Reines,D. and Warren,S.T. (2006) The absence of FMRP alters exon 15 FMR1 mRNA splicing in mouse cortical extracts. (A) RT–PCR analysis of splicing events in exon 15 using primers F13 and R15, hybridizing in exons 13 and 15, respectively. The ethidium bromide stained PCR products separated on a 1.8 % agarose gel is shown (negative image). 1, no Taq polymerase control; 2, no RT control with Wt total cortical RNA; 3, Wt total cortical RNA; 4, KO total cortical RNA; 5, Wt total RNA from cortical synaptoneurosomes; 6, KO total RNA from cortical synaptoneurosomes; 7, control pTL1 plasmid; L, DNA ladder. Expected size of each PCR product is indicated on the right side of the gel. (B) Densitometric analysis of RT–PCR products shown in (A) and expressed as the ratio of exon 15 isoforms for lanes 3, 4, 5 and 6, with the same color code as for splice scheme in (A). Error bars are standard deviations (KO) corresponds to cross-reactivity with FXRs. (C) Western blot analysis of FMRP expression in the cortical extracts. The band seen with anti-FMRP antibody in FMR1−/− (KO) corresponds to cross-reactivity with FXRs. (D) RT–PCR using primers F2-3 and R4, hybridizing over exons 2, 3 and 4, respectively. Samples tested are the same as in (A). (E) RT–PCR using primers F-GAPDH and R-GAPDH hybridizing in GAPDH with the same samples as in (A).

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