The gene encoding the fragile X RNA-binding protein is controlled by nuclear respiratory factor 2 and the CREB family of transcription factors

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The gene encoding the fragile X RNA-binding protein is controlled by nuclear respiratory factor 2 and the CREB family of transcription factors

Karen T. Smith, Robert D. Nicholls and Daniel Reines

ABSTRACT

FMR1 encodes an RNA-binding protein whose absence results in fragile X mental retardation. In most patients, the FMR1 gene is cytosine-methylated and transcriptionally inactive. NRF-1 and Sp1 are known to bind and stimulate the active, but not the methylated/silenced, FMR1 promoter. Prior analysis has implicated a CRE site in regulation of FMR1 in neural cells but the role of this site is controversial. We now show that a phospho-CREB/ATF family member is bound to this site in vivo. We also find that the histone acetyltransferases CBP and p300 are associated with active FMR1 but are lost at the hypoacetylated fragile X allele. Surprisingly, FMR1 is not cAMP-inducible and resides in a newly recognized subclass of CREB-regulated genes. We have also elucidated a role for NRF-2 as a regulator of FMR1 in vivo through a previously unrecognized and highly conserved recognition site in FMR1. NRF-1 and NRF-2 act additively while NRF-2 synergizes with CREB/ATF at FMR1's promoter. These data add FMR1 to the collection of genes controlled by both NRF-1 and NRF-2 and disfavor its membership in the immediate early response group of genes.

INTRODUCTION

Fragile X syndrome results from the loss of functional FMR1 gene product. This protein, FMRP, binds brain mRNA and therefore is important in nucleic acid metabolism [for a review see (1)]. In almost all fragile X patients, disease is due to transcriptional silencing of FMR1 which occurs after expansion of a trinucleotide repeat in the 5'-untranslated region (5'-UTR) of FMR1. Expansion triggers aberrant and permanent DNA methylation and heterochromatin formation and yields an FMR1 promoter that lacks its activators (2–6). Demethylation of FMR1 can be achieved in fragile X cell lines by treatment with 5-azadeoxycytidine which restores transcription factor function and an active state of chromatin (7–11).

In designing strategies to reactivate FMR1 transcription, not only it is important to understand the functions of the silencing factors, but also it is crucial to know the identities and contributions of the transcription factors and co-activators normally controlling FMR1. Silenced FMR1 in fragile X syndrome is marked by a loss of histone acetylation, compared with the active FMR1 gene (8). However, it is not known which proteins are responsible for normally maintaining histone acetylation at active FMR1. In addition, significant questions regarding the exact identities and functions of the transcription factors controlling FMR1 are still unanswered.

Four in vivo footprints were initially identified in the FMR1 promoter in primary and transformed cells of fibroblast and lymphoblast origin (12,13). Several proteins can bind the FMR1 promoter in vitro, including CREB, AP-2, Sp1, nuclear respiratory factor 1 (NRF-1) and upstream stimulatory factors 1 and 2 (henceforth referred to collectively as USF) (14–17). However, these initial studies have left unanswered questions regarding FMR1 transcription in vivo. We have previously shown that Sp1, NRF-1, USF and Max are bound specifically to the active FMR1 promoter in vivo and that Sp1 and NRF-1 were strong synergistic activators of FMR1 transcription (18). Recent work by others has shown that AP-2α may serve as a positive regulator of FMR1 transcription during development (19). Not all transcription factors bound at FMR1 in vivo are positively acting. In addition to Max-mediated repression of FMR1 transcription, we found that USF was unable to activate an FMR1-driven reporter in Drosophila SL2 cells, and indeed, strongly repressed Sp1 and NRF-1 mediated activation of...
FMRI (18). In addition, repression was not dependent on the E-box (18). This result was surprising, since mutation of the E-box has been shown to lower FMRI transcription by ~75% in PC12 cells and it has been speculated that USF operates through this site in vivo (16).

Therefore, it is still unclear which proteins are actually acting through the initially identified footprint over the E-box, which lies nearest to the transcription start site. It has been especially confusing since early experiments showed that a potential cAMP-responsive element (CRE) overlaps this E-box, suggesting that CREB may transduce neuronal stimuli and thereby impact FMRI transcription in brain (14). Despite early evidence, most subsequent reports have either not addressed this issue or obtained data that does not support this finding. For example, two in vivo footprinting reports showed data suggestive of proteins binding the E-box; however, both reports indicated that the partly overlapping CRE site was not occupied albeit neither examined neuronal cell types (12,13). In addition, EMSA analyses with an FMRI promoter fragment and nuclear extract failed to show that CREB could bind this site (16). Instead, USF bound in vitro to an FMRI promoter fragment and it was suggested that the E-box could be occupied by this factor in vivo (12,13,16). Furthermore, there is no evidence that CREB occupies the FMRI promoter in normal cells nor has a functional histone acetyltransferase been identified for this highly acetylated portion of chromatin.

Since our experiments with USF in vivo contradicted the proposed function that USF transactivates FMRI, we have now examined both CREB and USF at FMRI in human cells. USF represses FMRI reporter activity in HeLa cells suggesting that USF does not naturally regulate FMRI transcription through the E-box. Instead, we show occupancy of the active, but not inactive promoter, by the CREB/ATF family of transcription factors in vivo as well as the histone acetyltransferases CBP and p300. Surprisingly, we find that the endogenous FMRI gene in PC12 neuronal-like cells is not cAMP-inducible. We also learned that nuclear respiratory factor 2 (NRF-2) is bound to FMRI in normal but not patient cells and can transactivate the promoter. This is the first implication of this site or transcription factor at FMRI and is congruent with earlier evidence that NRF-1 is known to operate with NRF-2 to coordinate control of the transcription of genes involved in mitochondrial respiration and biogenesis (20). These data strongly suggest that CREB/ATF family members and not USF regulate FMRI transcription through the E-box/CRE, and together with NRF-2, positively contribute to FMRI expression. These findings lend new insight into the regulatory network to which the important RNA-binding protein, FMRP, belongs.

MATERIALS AND METHODS

Plasmid construction

Construction of pFMR1-luc, which contains a human FMRI promoter fragment (~272 to +291) driving firefly luciferase expression and mutation of the E-box/CRE in the FMRI promoter have been described previously (18). Mutation of the NRF-2 site in pFMR1-luc was performed by site-directed mutagenesis with the following oligos: 5’-CTTTGTTTGTTTTGTTACGTTGGAGGC-3’ and 5’-CGCCTCCACCGTTACGAAACCGAAACG-3’ which introduce a HpaI site into the NRF-2 binding site; the mutation was confirmed by sequencing. pACTIN-FL-NRF-1 contains full-length human NRF-1 with an N-terminal FLAG tag and pRL-dA5C contains the Renilla luciferase gene; both constructs are driven by the Drosophila actin 5C promoter and were constructed as described previously (18). A vector expressing full-length rat CREB was a gift from Dr Jerry Boss. Vectors expressing full-length human USF1 (pSV-USF1) or mouse USF2 (pSV-USF2) were gifts from Dr Michele Sawadogo (21). Dominant negative USF (A-USF) expression vector and empty control were gifts from Dr Charles Vinson (22,23). Vectors encoding full-length human NRF-2-alpha (A5CΔGABPα), NRF-2-beta (A5CΔGABPβ) and ATF-1 (A5CΔ-ATF1) for expression in Drosophila cells, were gifts from Dr Hiroshi Handa (24,25). A luciferase reporter construct containing the human HO-1 promoter (pHOGL3/4.5) was a gift from Dr Anupam Agarwal (26).

Cell culture and transfections

HeLa, SL2 and lymphoblastoid cell culture was carried out as described previously (18). Lymphoblastoid cell lines from a normal male (J-1) and a fragile X male patient (GM3200A) were obtained from Coriell Cell Repositories. J-1 contains an unmethylated 30 CGG repeat allele and GM3200A carries a methylated 530 CGG repeat allele as determined by methyl-sensitive restriction digest and Southern blot analysis (data not shown). PC12 cells were grown in DMEM (Cellgro) with 10% horse serum (Gibco), 5% FetalClone I (Hyclone), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified atmosphere at 37°C. HeLa cell transfections were carried out using Lipofectamine Plus (Invitrogen) as described previously (18). Each sample contained 200 ng of either mutant or wild-type pFMR1-luc reporter and 50 ng of the Renilla luciferase reporter pRL-cytomegalovirus (CMV) (Promega). In addition, transfections with wild-type USF contained 250 ng of either pSV-USF1 or pSV-USF2 expression plasmids, or 125 ng of pSV-USF1 and 125 ng of pSV-USF2, or an equal amount of empty expression vector. Alternatively, 100 or 200 ng of A-USF or empty expression vector were included in transfections as indicated. SL2 transfections were performed using Effectene (QIAGEN) as described previously (18). Samples contained 250 ng of either mutant or wild-type pFMR1-luc reporter and 25 ng of the Renilla luciferase reporter pRL-cytomegalovirus (CMV) (Promega). In addition, transfections with wild-type USF contained 250 ng of either pSV-USF1 or pSV-USF2 expression plasmids, or 125 ng of pSV-USF1 and 125 ng of pSV-USF2, or an equal amount of empty expression vector. Alternatively, 100 or 200 ng of A-USF or empty expression vector were included in transfections as indicated. SL2 transfections were performed using Effectene (QIAGEN) as described previously (18). Samples contained 250 ng of either mutant or wild-type pFMR1-luc reporter and 25 ng of pRL-dA5C. Transfections also included one or more of the following, as indicated in each figure: 300 ng of pACTIN-FL-NRF-1, 700 ng of NRF-2 which corresponds to 350 ng of each A5CΔ-hGABPα and A5CΔ-hGABPβ expression plasmids, 300 ng of ATF-1 expression plasmid (A5CΔ-hATF1) or empty vector. Where necessary, empty expression vector was added to keep total DNA levels constant between samples.

Antibodies, chromatin immunoprecipitation (ChIP) and forskolin treatment

Antibodies to CBP (A-22), p300 (C-20) and GABP-alpha (H-180) were purchased from Santa Cruz Biotechnology. Phospho-CREB antiserum was a gift from Dr Jerry Boss. ChIP in lymphoblastoid cells was performed as described previously (18). Each sample contained 200 ng of either mutant or wild-type pFMR1-luc reporter and 50 ng of the Renilla luciferase reporter pRL-cytomegalovirus (CMV) (Promega). In addition, transfections with wild-type USF contained 250 ng of either pSV-USF1 or pSV-USF2 expression plasmids, or 125 ng of pSV-USF1 and 125 ng of pSV-USF2, or an equal amount of empty expression vector. Alternatively, 100 or 200 ng of A-USF or empty expression vector were included in transfections as indicated. SL2 transfections were performed using Effectene (QIAGEN) as described previously (18). Samples contained 250 ng of either mutant or wild-type pFMR1-luc reporter and 25 ng of pRL-dA5C. Transfections also included one or more of the following, as indicated in each figure: 300 ng of pACTIN-FL-NRF-1, 700 ng of NRF-2 which corresponds to 350 ng of each A5CΔ-hGABPα and A5CΔ-hGABPβ expression plasmids, 300 ng of ATF-1 expression plasmid (A5CΔ-hATF1) or empty vector. Where necessary, empty expression vector was added to keep total DNA levels constant between samples.
transcription start site (61).

sequence of the overlapping E-box/CRE is shown underneath the site and that of the E-box/CRE mutant used in reporter assays is shown below the wild-type sequence.

5-primers were 5'-CTACATGCGAGGTTCAAGAC-3' and 5'-GAGGAAGCTCACCACGAG-3'. For the rat FMR1 promoter, primers were 5'-CTCTTTTGCCGATCGAG-3' and 5'-GGCCATAGCACTCAGCC-3'. For GABP-alpha ChIPs, primers used to amplify the human Tfam promoter were 5'-CGAGCTCCAGGCCTGCTGAAC-3' and 5'-CCTCGCGGGGAGGAATAAGAGC-3' and primers for the human FMR1 promoter were 5'-CTACGGGTACACAAAGGCTTGGTC-3' and 5'-GCAGTCGTACTGAGCGAGGGTTGAG-3'. For CBP, p300 and P-CREB ChIPs, primers for the rat c-fos promoter were 5'-CTCTTGATGGATGATGACCATC-3' and 5'-GCATTTGCTC-3' and primers for the human FMR1 promoter were 5'-CGACCTCATCCATCTGCA-3' and 5'-CCTCGCGGGGAGGAATAAGAGC-3' and primers for the human FMR1 promoter were 5'-GCTTGTTATAAAGCGAGGTACTGAG-3' and 5'-ATGTAATCCAGCAGGTCAGCAAAG-3'.

Quantitative RT-PCR

Quantitative PCR was performed on a BioRad iCycler using SYBR green. Cycling parameters were 95°C for 3 min, 46 cycles of 95°C for 15 s and 64°C (c-fos, FMR1 and Tfam promoters) or 60°C (FMR1, c-fos and HPRT transcripts) for 50 s, then 1 cycle of 95°C for 50 s and 55°C for 50 s. Primers to amplify the rat FMR1 transcript were 5'-GCTTGTTATAAAGCGAGGTACTGAG-3' and 5'-ATGTAATCCAGCAGGTCAGCAAAG-3'.

RESULTS

USF represses FMR1 promoter activity in mammalian cells

We have previously shown that USF represses FMR1 reporter activity in Drosophila SL2 cells (18). This result was unexpected, as mutation of the site through which it has been proposed to operate, the E-box/CRE (Figure 1), lowers expression to about 20% of normal in HeLa cells indicating it operates in a positive manner (Figure 2A). This is similar to prior observations in rat PC12 cells (16), and led us to explore the role of USF at FMR1 in human cells. We found that overexpression of USF1, USF2 or a combination of both, repressed FMR1 reporter activity in HeLa cells between 45 and 60% and repression was largely independent of the E-box (Figure 2B). To rule out the trivial possibility that this repression was due to overexpression of USF, we employed a dominant negative USF (A-USF) that binds the endogenous USF proteins and prevents them from interacting with DNA (22). Overexpression of A-USF in HeLa cells did not change FMR1-luc expression although the HO-1 promoter, previously shown to be controlled by USF, was repressed in a dose dependent manner (Figure 2C) (26). These results suggest that USF does not normally contribute in a positive way to FMR1 core promoter activity in HeLa cells.

FMR1 is bound in vivo by a phosphorylated CREB/ATF family member, as well as CBP/p300, but is not cAMP-inducible

Since our results suggested that USF is not responsible for the previously described footprint over the E-box, we turned our attention to a potential CRE in the human FMR1 promoter.
Figure 1. USF does not regulate transcription through the E-box. (A) HeLa cells were transfected with wild-type pFMR1-luc or with pFMR1-luc with point mutations in the E-box/CRE and luciferase activities were measured in cell lysates. (B) Either the wild-type (left) or E-box/CRE mutant reporter (right) was transfected into HeLa cells with or without pSV-USF1 and/or pSV-USF2 expression vectors as indicated. (C) pFMR1-luc or pHOGL3/4.5 was transfected into HeLa cells with the indicated amount of A-USF expression plasmid, or empty vector. Values for the FMR1 or HO-1 promoters were normalized to a CMV transfection control reporter. Data are expressed as percent normalized luciferase activity. Error bars represent ±1 standard deviation.

Identification of a conserved consensus NRF-2 binding site in the FMR1 5'-UTR

Previous FMR1 promoter analysis identified several protein binding sites conserved among species (16). Using homology alignment, we found another evolutionarily conserved transcription factor binding site that had not been previously identified (Figure 5A). The conservation of the GTTT sequences 5' to the boxed NRF-2 site suggests these bases are also important NRF-2 contacts (Figure 5A). Since recent work has shown...
that expression patterns of FMR1-encoded proteins in zebrafish, as well as Xenopus, are conserved compared to humans, we looked to see if the promoter sequences for FMR1 may also be conserved in these animals (27,28). We have now extended the alignment of the FMR1 5' region to include sequences from Danio rerio, Xenopus tropicalis as well as Gallus gallus (Figure 5A). A TATA-like motif and the NRF-2 site are conserved among all species tested, despite variations in the intervening sequence (Figure 5A). In addition, recently available genomic sequence of the zebrafish FMR1 gene shows that, in addition to the TATA-like and NRF-2 elements, sites representing the four in vivo footprints in the human FMR1 promoter are also conserved (Figure 5B).

The NRF-2 site is positively acting and NRF-2 stimulates the FMR1 promoter

We tested the function of the putative NRF-2 site in the human FMR1 promoter. Mutating four bases in the binding site (Figure 5A) reduced FMR1 promoter activity to 40% of wild-type in HeLa cells showing the importance of this site for FMR1 expression (Figure 6A). To test if FMR1 was a bona fide NRF-2 target, we asked if NRF-2 was bound to active FMR1 in human cells. Using ChIP and an antibody against its DNA-binding (alpha) subunit, we found that NRF-2 is bound to FMR1 in vivo (Figure 5A). As expected for an activator of transcription, NRF-2 was not bound at the silenced, expanded FMR1 allele in patient cells, although it occupies the active Tfam promoter in these cells (Figure 6B).

We have previously shown the importance of NRF-1 in FMR1 transcription (18). NRF-1 often works at promoters with NRF-2; this is especially well documented for numerous nuclear-encoded mitochondrial genes (20,29). To test NRF-2’s transcriptional activity at FMR1, we asked whether NRF-2 could activate the FMR1 promoter in SL2 cells which have previously proven successful in assessing the function of this transcription factor (25,30–32). As expected, transfection of either NRF-2-alpha or NRF-2-beta alone had no effect on FMR1 transcription (data not shown). However, cotransfection of both subunits activated the FMR1 promoter between 1.7- and 3.1-fold (Figure 7A and B). We then tested if NRF-1 and NRF-2 could work together at FMR1 and found that they activated transcription additively (Figure 7A). Since members of the CREB/ATF family have been shown to work with NRF-2 to synergistically activate transcription, we wanted to test if this occurred at FMR1 (25). Indeed, we found that ATF-1 activates FMR1 transcription synergistically with NRF-2 (Figure 7B). This result is similar to that obtained with CREB and NRF-2 (data not shown). Importantly, mutation of either the E-box/CRE or the NRF-2 binding site abolished activation by ATF-1 and NRF-2 (Figure 7B).

DISCUSSION

The precise roles and relative importance of the many transcription factors implicated in FMR1 promoter function is controversial. Early reports suggested that FMR1 is regulated by cAMP signaling, perhaps through CREB (14). Here we show that CRE-binding proteins play a role in FMR1 transcription, however the endogenous gene is not induced in a manner such as that observed for the immediate early c-fos gene. These data are consistent with our findings that USF does not appear to operate through this region of the promoter resolving the paradox that a protein resides at this site but that USF does not activate this promoter. It is also worth noting that Drosophila SL2 cells accurately predicted how USF
proteins would act at the *FMR1* promoter in a human cell line, showing that this is a useful cell line for studying these human transcription factors (18). Due to the overlapping nature of the E-box and the CRE, it is unlikely that both could be occupied at the same time by different transcription factors. However, we cannot rule out the possibility that this overlapping site could be differentially used by E-box binding factors and CRE-binding proteins in different cell types. To date, there is no direct *in vivo* evidence that USF is positively acting through the E-box/CRE site at *FMR1* in any cell type and c-Myc is not present at *FMR1* *in vivo* though other E-box binding proteins could play a role (18). *In vitro*, USF can bind to an *FMR1* promoter fragment and induce bending of an *FMR1* promoter fragment (16,17). Curiously, our previous
in vivo ChIP results indicate that USF resides on the FMR1 promoter in lymphoblastoid cells however it does not regulate the FMR1 promoter through the E-box (18). USF has also been shown to work through initiator (Inr) elements that surround the transcription start site (33). However, mutation of an Inr element in FMR1 did not affect promoter activity in reporter assays (16). The recognition site for USF and its function at FMR1 remain to be elucidated. Our data suggest that USF is not acting in the ~C24300 bp upstream of the transcription start site and no incriminating in vivo footprints were identified up to ~C24600 bp upstream of the start site, although there is a perfect consensus E-box at ~C24800 bp from the transcription start (12,13). If bound by USF, perhaps this site plays a role in maintaining chromatin structure of FMR1 on the endogenous locus and hence, its role, as determined using a minimal promoter fragment on reporter plasmids and through in vitro assays, has not been appreciated. Consistent with this idea, USF has recently been implicated in recruiting histone acetyltransferase activity at genomic regions suggesting that these proteins could play a similar role at FMR1 (34). In addition to clarifying which protein is working through the E-box/CRE, we have found a new transcription factor binding site on FMR1 that binds NRF-2/GA-binding protein (GABP) in vivo. This site has remained unidentified as previous footprinting analyses either did not cover this region (13) or did not find evidence of a footprint in this area (12). Perhaps the binding of NRF-2 to this site is not stable enough to be detected reliably without the use of crosslinking agents. It has been previously shown that NRF-2 binding is affected by methylation of its recognition site (35–37). Indeed, our results show that NRF-2 does not occupy the promoter in patient cells, thereby reinforcing the view that a global repression mechanism shuts this gene off in patients. A role for NRF-2 at FMR1 follows from our previous evidence showing the importance of NRF-1 in FMR1 expression. In addition to its role at FMR1, potential NRF-1 sites have been reported in the promoters of multiple other genes involved in translation and mRNA metabolism, e.g. eIF2-alpha, eIF2-beta and hnrNP-A2 (12,38), and we have discovered putative NRF-2 binding sites in all of these promoters as well (data not shown). Thus, FMRP could fit into a ‘RNA metabolism’ regulon of genes controlled by both NRF-1 and NRF-2, since it is an RNA-binding protein associated with translation (1).

Figure 5. Identification and conservation of a NRF-2/GABP binding site in the 5′-UTR of FMR1. (A) Sequence alignment of FMR1 homologs from several species showing a newly identified binding site for NRF-2. GenBank accession nos are as follows: L29074.1 (Homo sapiens), AY630337 (Rattus norvegicus), AY630338 (Mus musculus), AF251348 (Canis familiaris), AF251350 (Macaca arctoides), AF251349 (Pan troglodytes), NM_152963 (D. rerio), BC074570 (X. tropicalis) and CR386208 (G. gallus). The major transcription start site as identified in humans is shown and numbering is based on the human FMR1 sequence (61). Nucleotides conserved in all nine species are indicated with an asterisk. The underlined bases in the NRF-2 human sequence represent those changed for mutational analysis and the changes are shown in italics above the site. (B) Identification of the zebrafish FMR1 promoter (GenBank accession no. BX005284) and alignment with the human promoter (accession no. L29074.1). Boxes represent protein binding sites known to operate in humans that are conserved in zebrafish. Underlined sites represent potential positional differences between the two species for these factors. Conserved nucleotides are denoted with an asterisk.
reports that have tested this hypothesis on the endogenous \textit{FMR1} gene. Phosphorylation of CREB was originally thought to be specific and sufficient for the cAMP-induction of genes. Recent work, however, shows that genes can be bound by NRF-2 is a regulator of \textit{FMR1} transcription \textit{in vivo}. (A) HeLa cells were transfected with wild-type or NRF-2 site mutant pFMR1-luc reporter plasmids. Values for the \textit{FMR1} promoter were normalized to a CMV transfection control reporter. Data plotted are the average of six independent transfections ±1 standard deviation. The wild-type reporter was arbitrarily set to 100%. (B) Immunoprecipitation using an anti-NRF-2 antibody or non-specific IgG (N.S.) was performed on chromatin from normal and fragile X cells. Real-time PCR was carried out on triplicate IPs to quantify the binding of NRF-2 to the \textit{Tfam} and \textit{FMR1} promoters; error bars represent ±1 standard deviation.
P-CREB regardless of cAMP-inducibility (40). Indeed, we have shown that even though FMR1 is bound by P-CREB/ATF as well as by CBP/p300, it is not cAMP-inducible. An important step in CREB/ATF function is the recruitment of CBP/p300 to P-CREB (41). Sp1, NRF-1 and NRF-2, may also play a role in recruiting CBP/p300 suggesting that these HATs may be recruited to FMR1 through their interactions with multiple transcription factors (42–47).

Interestingly, the overlapping E-box and CRE elements in the FMR1 promoter are almost identical to the E-box/CREs site in the human c-fos promoter with a one base pair difference in the E-box: CCGTGACGT versus FMR1’s: CACGTTACG. However, we have shown that there are many differences between the two genes and their regulation by CRE-binding proteins. The co-existence of a TATA box and a CRE within a promoter has been shown to correlate with cAMP-responsiveness (48). The absence of a canonical TATA box from the FMR1 promoter may partly explain its non-responsiveness to cAMP. In this regard, FMR1 has many characteristics of a housekeeping gene, including the lack of a TATA box, a high GC content and its regulation by factors such as NRF-1, NRF-2 and Sp1 (49, 50). The prior observation that an FMR1-driven reporter construct was cAMP-inducible, may have resulted from the inclusion of only a small fragment of the FMR1 promoter, which contained the CRE but lacked the other FMR1 transcription factor binding sites (14).

FMRP has been implicated in synaptic plasticity suggesting that, at some level, FMR1 expression may be responsive to neuronal signals (51–53). However, FMR1 transcription was not responsive to cAMP, or to depolarization which is known to activate NRF-2 (54, 55). Perhaps FMR1 does not need to be induced at the transcriptional level in adult neurons since post-translational changes modulate FMRP in response to certain stimuli (56–58). For example, FMR1 mRNA and FMRP rapidly localize in dendrites in response to treatment with KCl and FMRP is quickly translated within 5 min of mGlur stimulation of neurons (56, 57). Even though these stimuli activate CREB, it would take longer to make FMRP through a transcriptional induction pathway than to directly increase FMRP levels by post-transcriptional processes (59, 60). Hence, mobilization of pre-existing FMR1 mRNA and protein, along with rapid local translation may be more important for the neuronal functions of FMRP than new mRNA synthesis. In support of this model, FMRP levels increase in cortical neurons following stimulation by light exposure, but mRNA levels do not change (58). A complete description of the regulatory landscape of the FMR1 promoter will facilitate the examination of the intrinsic developmental and external neuronal signaling mechanisms by which regulation of FMR1 is achieved.

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