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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

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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...
and thereby impact E-box, suggesting that CREB may transduce neuronal stimuli that a potential cAMP-responsive element (CRE) overlaps this has been especially confusing since early experiments showed that E-box, which lies nearest to the transcription start site. It acting through the initially identified footprint over the E-box has been shown to lower FMR1 (18). This result was surprising, since mutation of the E-box has been shown to lower FMR1 transcription in vivo (16). However, both reports indicated that the partly overlapping CRE site was not occupied albeit neither studied neuronal cell types (12,13). In addition, EMSA analyses with an FMR1 promoter fragment and nuclear extract failed to show that CREB could bind this site (16). Instead, USF bound in vitro to an FMR1 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 FMR1 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 FMR1, we have now examined both CREB and USF at FMR1 in human cells. USF represses FMR1 reporter activity in HeLa cells suggesting that USF does not naturally regulate FMR1 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 FMR1 gene in PC12 neuronal-like cells is not cAMP-inducible. We also learned that nuclear respiratory factor 2 (NRF-2) is bound to FMR1 in normal but not patient cells and can transactivate the promoter. This is the first implication of this site or transcription factor at FMR1 and is congruent with earlier evidence that NRF-1 is known to operate with NRF-2 to coordinately control the transcription of genes involved in mitochondrial respiration and biogenesis (20).

These data strongly suggest that CREB/ATF family members and not USF regulate FMR1 transcription through the E-box/CRE, and together with NRF-2, positively contribute to FMR1 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 FMR1 promoter fragment (~272 to +291) driving firefly luciferase expression and mutation of the E-box/CRE in the FMR1 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’-CGTTTCGTTTCAACCTGAGGGCC-3’ and 5’-GCCCTTACCGTTAACGAAACGGGAC-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 (A5CΔ-GABPα), NRF-2-beta (A5CΔ-GABPβ) 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). 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).
previously (18). ChIP in PC12 cells was done essentially the same way, with a few exceptions. PC12 cells at ~60–80% confluency were grown in DMEM containing 0.5% FetalClone I (HyClone) and treated the next day with 10 μM forskolin (Sigma) or an equal volume of dimethyl sulfoxide (DMSO) for 25 min. Formaldehyde was added to the media to 20 min and whole cell extracts were prepared for ChIP. For the rat c-fos promoter, primers were 5'-CCTACATGCGAGGGTTCACAGAC-3' and 5'-GAGTTAGTGAGCGGCTCTATCAGC-3'. For the rat FMR1 promoter, primers were 5'-CTCTTGGCGCATGACACC-3' and 5'-GAGCGATAGCGGACAC-3'. For GABP-alpha ChIPs, primers used to amplify the human Tфам promoter were 5'-CGAGCTCCAGCCCTGCTGAAC-3' and 5'-CCTCGGGGGGAG-GAAATAGAGC-3' and primers for the human FMR1 promoter were 5'-CTACGCGGTACACAAAAGCCTGGTGTCAC-3' and 5'-GCAGTGCTCGACTGACGAGGTCG-3'. For CBP, p300 and P-CREB ChIPs, primers for the human c-fos and FMR1 promoters were 5'-CAGGCAGGTTCGGT-3' and 5'-ATGTAATCCAGCAGGTCAGCAAAG-3'. Tran

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. Human FMR1 promoter map showing the four binding sites known to be occupied in vivo and the identities of the likely factors at those sites (12,13). The 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 with mutated bases underlined. A question mark above the E-box/CRE represents the unknown factor(s) binding this site in vivo. The bent arrow indicates the major transcription start site (61).
(Figure 1) which overlaps the E-box and is well conserved across species (12–14,16). It was initially shown that recombinant CREB can bind an FMR1 promoter fragment in vitro and that overexpression of CREB could increase activity from an FMR1-driven reporter (14). However, subsequent reports suggested that the CRE is not involved in FMR1 transcription, though none directly test CREB’s role in vivo (12,13,16).

Since our results suggested that USF is not acting through the E-box, we tested the idea that CRE-binding proteins reside at this site.

Since initial experiments suggested that a CRE-binding protein could act at FMR1 and that this gene may be induced by the cAMP pathway, we employed ChIP using an antibody raised against CREB phosphorylated at Ser 133 (J. Lochamy and J. Boss, manuscript submitted). Indeed, we found that a phosphorylated CREB/ATF family member was bound at the active human FMR1 and c-fos promoters in lymphoblastoid cells, but not at silenced FMR1 in fragile X cells (Figure 3A). This was specific for FMR1, since P-CREB occupied the c-fos promoter in fragile X cells (Figure 3A). Since a P-CREB family member was bound at FMR1, we also tested if the histone acetyltransferases CBP and p300 that bind P-CREB were associated with active FMR1, which shows robust histone acetylation (8). Indeed, both histone acetyltransferases reside at FMR1 and their presence was largely reduced at the fragile X allele (Figure 3B).

We then used undifferentiated PC12 cells to test if the endogenous FMR1 gene is cAMP-inducible. Cells were treated with 10 μM forskolin or DMSO (vehicle). Real-time PCR of endogenous c-fos showed its strong induction (~50-fold) at 1 h post-stimulation (Figure 4A). However, FMR1 and HPRT transcript levels did not change (Figure 4A and data not shown). Even after 24 h, FMR1 mRNA levels remained constant (data not shown) revealing that in contrast to previous suggestions, the endogenous FMR1 gene is not cAMP-inducible in this cell type. We also obtained similar results after KCl/CaCl2 treatment of PC12 cells (data not shown).

Since it has been shown that at c-fos, the phosphorylation of CREB increases in response to forskolin treatment, and this is followed by induction of transcription, we tested if phosphorylation of CREB/ATF at FMR1 changed after forskolin treatment. We found that the CRE-binding proteins at FMR1 and c-fos were phosphorylated in undifferentiated PC12 cells without forskolin treatment (Figure 4B, upper panel). We performed quantitative analyses of P-CREB binding at c-fos and FMR1 and found that in response to forskolin treatment, the increase in occupancy of the phosphorylated CRE-binding protein on FMR1 was ~2.2-fold (Figure 4B, lower left panel), while that on c-fos was ~4.6-fold (Figure 4B, lower right panel).

**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 5B).

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, co-transfection 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 \textit{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 \textit{in vivo} evidence that USF is positively acting through the E-box/CRE site at \textit{FMR1} in any cell type and c-Myc is not present at \textit{FMR1} \textit{in vivo} though other E-box binding proteins could play a role (18). \textit{In vitro}, USF can bind to an \textit{FMR1} promoter fragment and induce bending of an \textit{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 /C24 300 bp upstream of the transcription start site and no incriminating in vivo footprints were identified up to /C24 600 bp upstream of the start site, although there is a perfect consensus E-box at /C24 800 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).

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.

The best studied functions for NRF-1 and NRF-2, however, are in controlling transcription of nuclear-encoded mitochondrial-related genes, notably the major transcription factor Tfam, as well as mitochondrial transcription specificity factors (20,39). It is not clear what separates or potentially links NRF-1 and NRF-2 mediated regulation of mitochondrial-related genes and those involved in RNA metabolism, or other functions.

An important question is the inducibility of FMR1 promoter function. Despite the suggestion made several years ago that FMR1 may be cAMP-inducible, we are not aware of any
reports that have tested this hypothesis on the endogenous 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...
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/CRE site in the human c-fos promoter with a one base pair difference in the E-box: CCCGTCACGT versus FMR1’s: CACGTGACGT. 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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