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Karen T Smith, *Emory University*
Robert D. Nicholls, *Children's Hospital of Pittsburgh*
Daniel Reines, *Emory University*

Journal Title: Nucleic Acids Research
Volume: Volume 34, Number 4
Publisher: Oxford University Press (OUP): Policy C - Option B | 2006-01-01, Pages 1205-1215
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1093/nar/gkj521
Permanent URL: https://pid.emory.edu/ark:/25593/tmscz

Final published version: http://dx.doi.org/10.1093/nar/gkj521

Copyright information:

Published online February 25, 2006

Nucleic Acids Research, 2006, Vol. 34, No. 4 ***1205–1215***
doi:10.1093/nar/gkj521

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[1] and Daniel Reines*

Program in Genetics & Molecular Biology and Department of Biochemistry, Emory University School of Medicine, 1510 Clifton Road, Atlanta, GA 30322, USA and [1]Birth Defects Laboratories and Division of Medical Genetics, Department of Pediatrics, Children's Hospital of Pittsburgh, 3705 Fifth Avenue, Pittsburgh, PA 15213, USA

Received November 15, 2005; Revised January 18, 2006; Accepted February 7, 2006

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

*To whom correspondence should be addressed. Tel: +1 404 727 3361; Fax: +1 404 727 3452; Email: dreines@emory.edu

FMR1 (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 *FMR1* 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 *FMR1* 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 study examined 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′-CGTTTCGGTTTCGTTAACGGTGGAGGGC-3′ and 5′-GCCCTCCACCGTTAACGAAACCGAAACG-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 (A5CΔP-GABPα), NRF-2-beta (A5CΔP-GABPβ) and ATF-1 (A5CΔP-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 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 A5CΔP-hGABPα and A5CΔP-hGABPβ expression plasmids, 300 ng of ATF-1 expression plasmid (A5CΔP-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). 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 for 20 min and whole cell extracts were prepared for ChIP. For the rat *c-fos* promoter, primers were 5′-CCTACATGCGGAGGGTCCAG-GAGAC-3′ and 5′-GAGTAGTAGGCGCCTCAGCTGGCC-3′. For the rat *FMR1* promoter, primers were 5′-CTCTT GGGCAGCTGAGCACGC-3′ and 5′-GGCCATAGGCAG-CACGACCTG-3′. For GABP-alpha ChIPs, primers used to amplify the human *Tfam* promoter were 5′-CGAGCTC-CAGCCCTGGCTTGAAC-3′ and 5′-CCTCGCGGGGGAG-GAATAAGAGC-3′ and primers for the human *FMR1* promoter were 5′-CTACGGGTCACAAAAGCCTGGGT-CACC-3′ and 5′-GCAGTCTGACTGAGCGGGAGGTG-GAG-3′. For CBP, p300 and P-CREB ChIPs, primers for the human *c-fos* promoter were 5′-GCTTGTTATAAAAG-CAGTGGCTGCG-3′ and 5′-GTTGAAGCCCGAGAACAT-CATCG-3′ and primers for the human *FMR1* promoter were 5′-CGACTCAATCCATGTCCCTTAAAGG-3′ and 5′-CCAGTTCGGCCTCTCTGGGATTCC-3′. Cycling conditions were 94°C for 2 min, 33 cycles of 94°C for 45 s, 64°C (rat and human *c-fos and FMR1* promoters) or 68°C (human Tfam and *FMR1* promoters) for 50 s and 72°C for 45 s, followed by 72°C for 7 min. PCR products were separated by gel electrophoresis through 1.5% agarose and bands were detected by ethidium bromide staining.

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′-GCATTT-GTAAAAGATGTCCATG-3′ and 5′-CAAGGCTCTTTTT-CATTTGCTC-3′, for the rat *c-fos* transcript, primers were 5′-GGAGCTGACAGATACGCTCCA-3′ and 5′-GATCTTG-CAGGCAGGTCGGT-3′ and for the rat *HPRT* transcript primers were 5′-CGTGATTAGTGATGATGAACCAGG-3′ and 5′-ATGTAATCCAGCAGGTCAGCAAAG-3′. Transcript levels for *FMR1* and *c-fos* were calculated from a standard curve and normalized to *HPRT* mRNA levels. Primers for real-time PCR of the rat *c-fos* promoter were 5′-CCTACATGCGGAGGGTCCAGGAGAC-3′ and 5′-CCCTGTCATCAACTCTACGCCCCA-3′. Primers for real-time PCR of the rat *FMR1* promoter were 5′-CCAGCG-CAGTGAAAAATGTACC-3′ and 5′-GTACAGGAGGAA-GCGAGGACG-3′. For the human *FMR1* promoter, primers were 5′-GGAATGTAAAGGGTTGCAAGGAGG-3′ and 5′-CACTTGAGGTTCATTTCTGCCCC-3′. Human *c-fos* promoter primers were 5′-GCTTGTTATAAAAGCAGT-GGCTGCG-3′ and 5′-GTTGAAGCCCGAGAACATCA-TCG-3′ and human *Tfam* promoter primers were 5′-GGTCCTGGATGCAGGACTGTCTG-3′ and 5′-GGAAA-CCGCAATCCTCTAGCCTGC-3′. Relative amounts of promoter DNA isolated after ChIP were calculated from a standard curve of input DNA for each promoter.

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

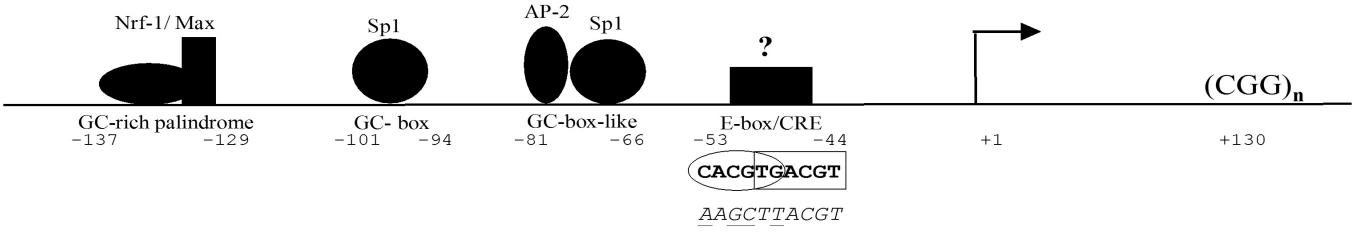

Figure 1. Human *FMR1* promoter map showing the four binding sites known to be occupied *in vivo* and the identities of the likely factors at those sites (12,13). The sequence of the overlapping E-box/CRE is shown underneath the site and that of the E-box/CRE mutant used in reporter assays is shown below 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).

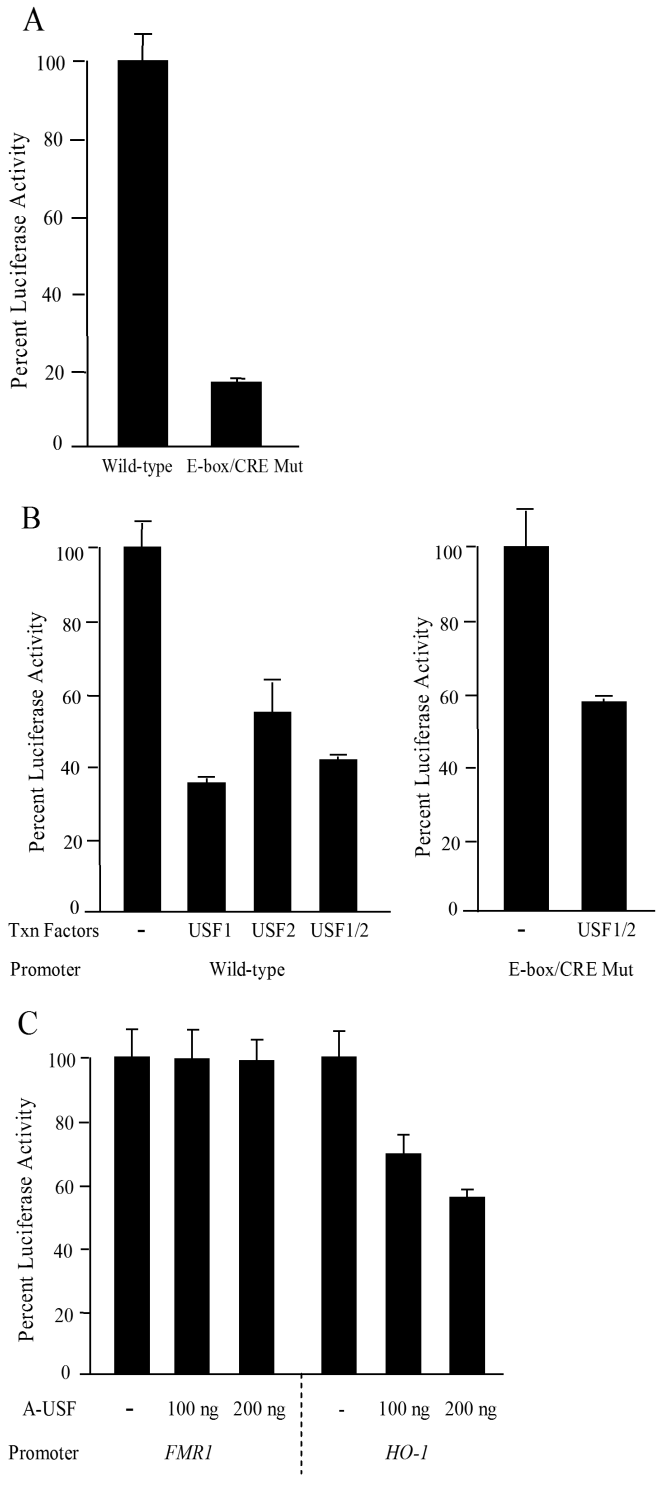

Figure 2. USF does not regulate transcription through the E-box. (**A**) HeLa cells were transfected with wild-type pFMR1-luc or with pFMR1-luc with point mutations in the E-box/CRE and luciferase activities were measured in cell lysates. (**B**) Either the wild-type (left) or E-box/CRE mutant reporter (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.

(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/$CaCl_2$ 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

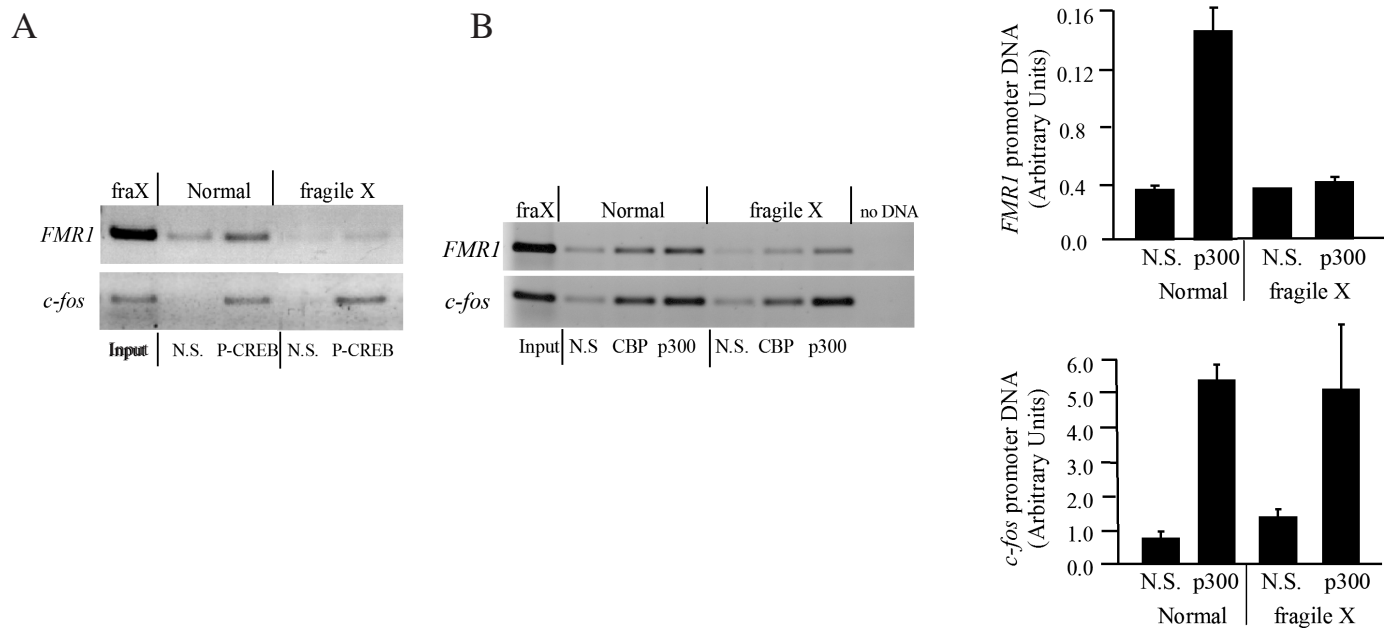

Figure 3. P-CREB/ATF and CBP/p300 occupy human *FMR1 in vivo*. (**A**) ChIP was carried out with either anti-P-CREB antibody or (**B**) antibodies to CBP and p300, or non-specific rabbit IgG [N.S. in both (A and B)]. PCR was performed to amplify the *FMR1* and *c-fos* promoters in normal and fragile X cells (left). Occupancy of p300 was quantified by real-time PCR of an independent set of triplicate IPs (right); error bars represent ±1 standard deviation.

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 6B). 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, 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

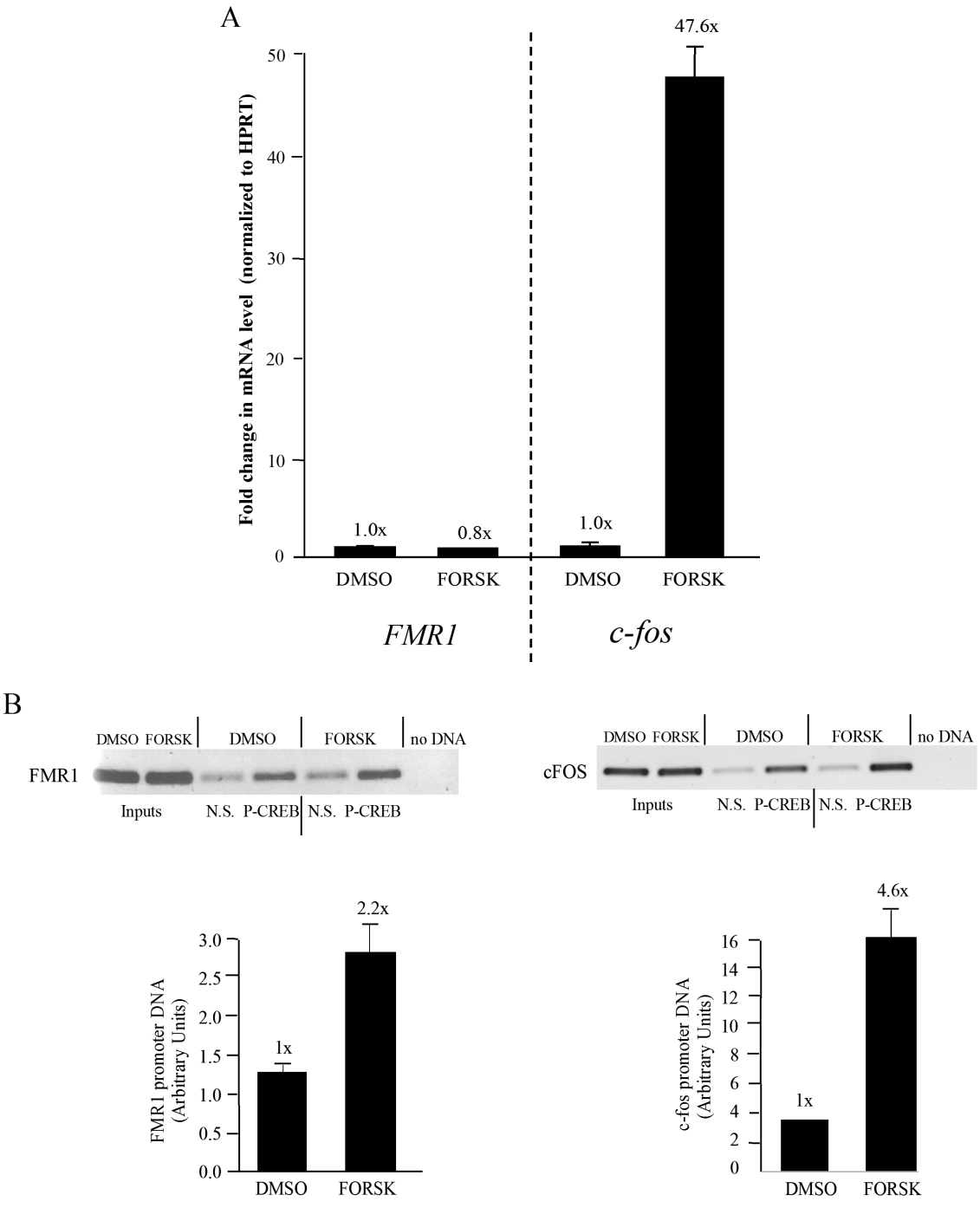

Figure 4. *FMR1* transcription is not cAMP-inducible. (**A**) Real-time PCR quantification of endogenous *FMR1*, *HPRT* and *c-fos* transcripts in PC12 cells with or without 1 h of forskolin stimulation. (**B**) Phosphorylation of CREB/ATF at *FMR1* increases 2-fold after forskolin induction of PC12 cells. ChIP was performed with P-CREB antiserum on DMSO- or forskolin-treated PC12 cells. PCR was performed to amplify the rat *FMR1* and *c-fos* promoters (top). Quantification of P-CREB binding was performed by real-time PCR (bottom). Error bars represent ±1 standard deviation. For *FMR1* real-time analysis, two independent IPs are represented, each with duplicate real-time PCR. For *c-fos*, the real-time data represent one IP with quadruplicate real-time PCR.

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

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.

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 ∼300 bp upstream of the transcription start site and no incriminating *in vivo* footprints were identified up to ∼600 bp upstream of the start site, although there is a perfect consensus E-box at ∼−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).

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).

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

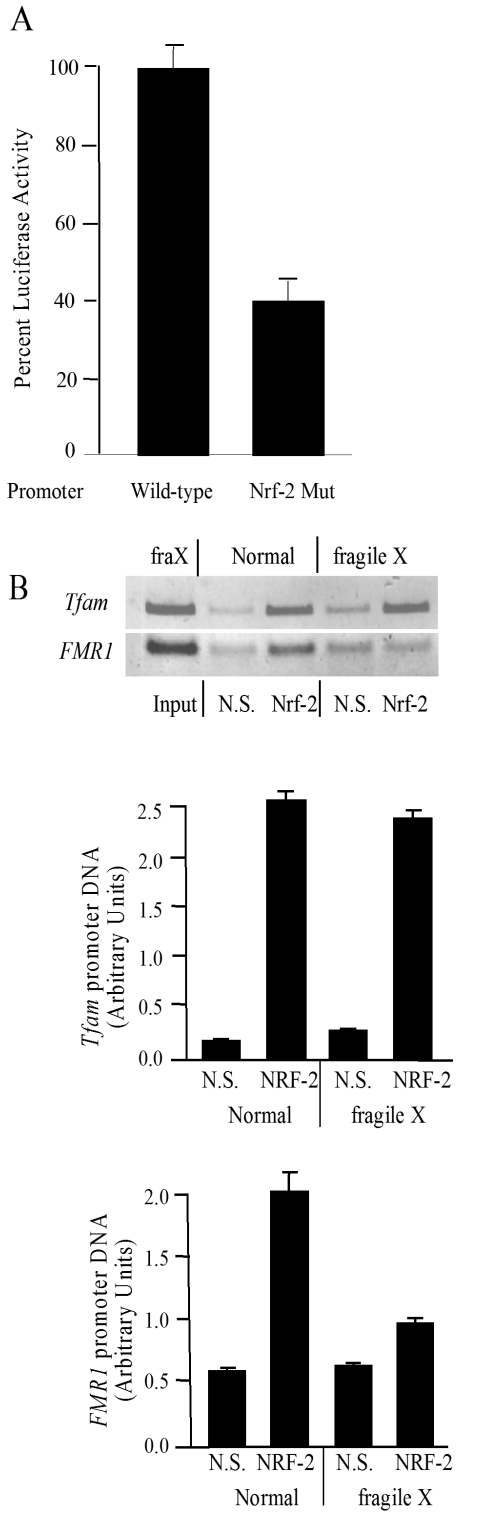

Figure 6. NRF-2 is a regulator of *FMR1* transcription *in vivo*. (**A**) HeLa cells were transfected with wild-type or NRF-2 site mutant pFMR1-luc reporter plasmids. Values for the *FMR1* promoter were normalized to a CMV transfection control reporter. Data plotted are the average of six independent 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 *Tfam* and *FMR1* promoters; error bars represent ±1 standard deviation.

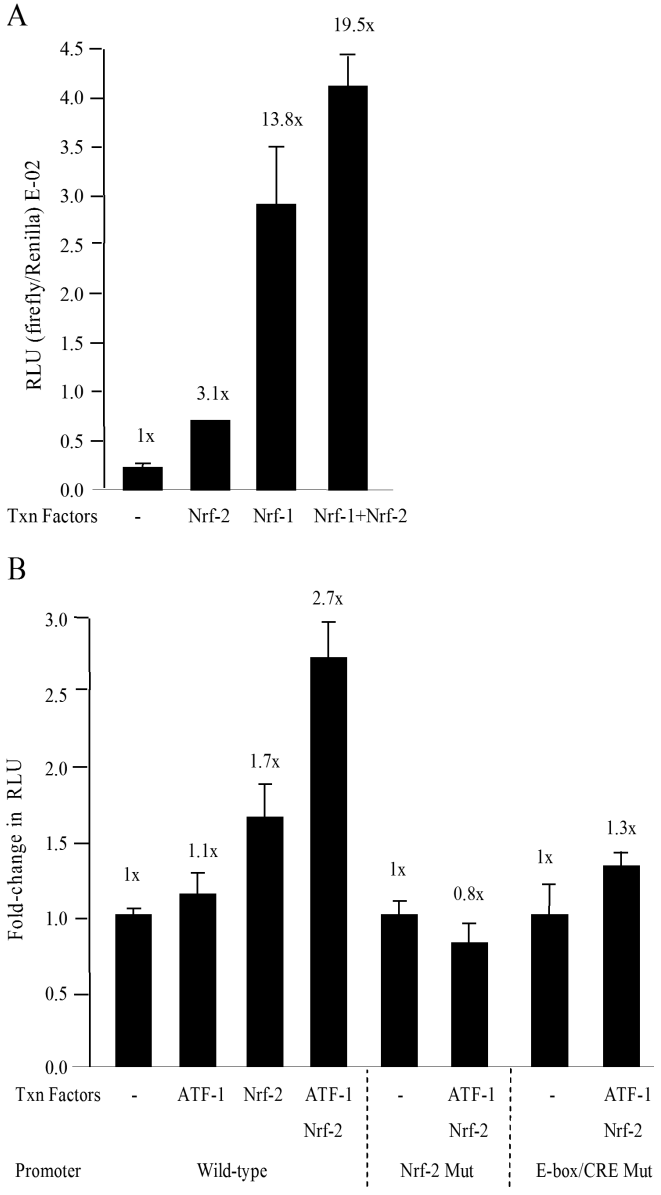

Figure 7. Stimulation of the human *FMR1* promoter in *Drosophila* SL2 cells. (**A**) pFMR1-luc reporter plasmid was co-transfected into SL2 cells with an empty vector or vectors expressing NRF-1 (pACTIN-FL-NRF-1), or NRF-2 (both A5CΔP-hGABPα and A5CΔP-hGABPβ) or both NRF-1 and NRF-2. Firefly luciferase values are normalized to a *Renilla* luciferase control. The fold-changes are shown above each bar in relation to the sample representing pFMR1-luc alone which was set to 1×. Each bar represents the average of three transfections and error bars represent ±1 standard deviation, except for NRF-2 for which the average of two transfections was plotted. (**B**) pFMR1-luc, or pFMR1-luc with the NRF-2 or E-box/CRE sites mutated, were co-transfected with vectors expressing human ATF-1 (A5CΔP-hATF1), or NRF-2 (both A5CΔP-hGABPα and A5CΔP-hGABPβ) or both ATF-1 and NRF-2. The fold-changes are shown above each bar, in relation to their appropriate controls and are plotted in relative light units (RLU) on the vertical axis. Bars represent the averages of at least three transfections. Error bars for all samples represent ±1 standard deviation.

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: CCCGTGACGT 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.

ACKNOWLEDGEMENTS

The authors thank Jonathan Lochamy and Dr Jerry Boss for the gift of phospho-CREB antiserum, CREB expression vectors, and helpful advice. The authors are grateful to Drs Hiroshi Handa, Michele Sawadogo, Charles Vinson and Anupam Agarwal for providing materials. The authors also thank Wesley Davis for plasmid construction. Supported by NIH grant HD35576. Funding to pay the Open Access publication charges for this article was provided by NIH.

Conflict of interest statement. None declared.

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