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Regulating Fragile X Gene Transcription in the Brain and Beyond

JAE H. LIM, ANNE B. BOOKER, AND JUSTIN R. FALLON*
Department of Neuroscience, Brown University, Providence, Rhode Island

The past several years have seen remarkable growth in our understanding of the molecular processes underlying fragile X syndrome (FXS). Many studies have provided new insights into the regulation of *Fmr1* gene expression and the potential function of its protein product. It is now known that the promoter elements modulating *Fmr1* transcription involve a complex array of both *cis* and *trans* factors. Moreover, recent studies of epigenetic modification of chromatin have provided novel clues to unlocking the mysteries behind the regulation of *Fmr1* expression. Here, we review the latest findings on the regulation of *Fmr1* transcription. J. Cell. Physiol. 205: 170–175, 2005. © 2005 Wiley-Liss, Inc.

Fragile X syndrome (FXS) is the most common form of inherited mental retardation with incidence of about 1:4,000 in males and 1:8,000 in females. Individuals with FXS display moderate to severe mental retardation and have IQs between 20 and 60. Other clinical features are highly variable and include craniofacial abnormalities, macroorchidism in post-pubescent males, connective tissue dysplasia, and hyperactive attention deficit disorder (reviewed in Hagerman and Hagerman, 2003). A massive trinucleotide (CGG) repeat-mediated transcriptional silencing of the *Fmr1* gene is the key molecular defect in FXS. Specifically, individuals carrying a *Fmr1* allele with 200 or more CGG repeats manifest the clinical phenotypes of FXS. The expansion of these trinucleotide repeats, which are located in the first exon of the gene, results in aberrant methylation of the nearby CpG islands in the promoter and in the repeat itself. This change is thought to interfere with the assembly of transcriptional (Smith et al., 2004) as well as epigenetic (Harikrishnan et al., 2005) complexes, leading to the suppression of *Fmr1* transcription. While the importance of this dysregulation of *Fmr1* transcription in FXS has been firmly established, little is known about the actual molecular elements mediating the expression of *Fmr1* in either normal or pathological states.

Since the initial identification of the *Fmr1* gene (Bell et al., 1991; Heitz et al., 1991; Vincent et al., 1991), much effort has been directed towards elucidating the molecular basis of FXS. *Fmr1* encodes FMRP, an RNA-binding protein that binds a unique set of mRNAs (Brown et al., 2001; Darnell et al., 2001; Denman, 2003; Sung et al., 2004). In neurons, FMRP has been suggested to play an important role in synaptic plasticity, perhaps by trafficking mRNAs to the dendrites (reviewed see O'Donnell and Warren, 2002). One strategy for understanding FMRP function is to systematically characterize the regulatory elements mediating the *Fmr1* transcription. This approach has been fruitful for characterizing the functions of a wide range of proteins (Werner, 2003).

Recent studies have identified several transcription factors as well as other *cis* and *trans* elements that bind to the *Fmr1* promoter (Drouin et al., 1997; Schwemmle et al., 1997; Carrillo et al., 1999; Kumari and Usdin, 2001; Smith et al., 2004). These observations, along with the findings that *Fmr1* expression is tightly controlled during development, suggest that a complex array of positive and negative factors act in concert to regulate *Fmr1* transcription. Epigenetic modifications such as the methylation of CpG sites in the *Fmr1* promoter and acetylation of histones have also been shown to play an important role in modulating the expression of both normal and mutated *Fmr1* gene (Chiurazzi and Neri, 2001; Harikrishnan et al., 2005; Pietrobono et al., 2005). However, the specific changes in the chromatin that lead to altered *Fmr1* expression remain to be elucidated.

Another insight into the regulation of *Fmr1* gene expression has come from studies examining carriers of *Fmr1* pre-mutation alleles (61–200 repeats). These individuals develop a distinct set of clinical features collectively known as the fragile X-associated tremor/ataxia syndrome or FXTAS (Tassone et al., 2000; Hagerman and Hagerman, 2004). In these individuals, FMRP levels are moderately reduced, but *Fmr1* transcript expression is elevated. These observations suggest that *Fmr1* transcript overexpression may be a toxic gain of function mutation. These findings also underscore that *Fmr1* mRNA levels are tightly regulated in normal cells, and disruption of this fine balance can manifest in the clinical phenotypes of FXS or FXTAS (Oostra and Willemsen, 2003). Undoubtedly, the identification of the gene regulatory elements and the underlying signaling pathways will provide deeper insights into the pathogenesis of FXS and FXTAS. A potentially beneficial transcriptional and/or epigenetic therapy to reactivate *Fmr1* gene expression can then be fully envisioned.

Fmr1 EXPRESSION DURING DEVELOPMENT

The expression pattern of *Fmr1* has been the subject of numerous reports. (reviewed in Hagerman and Hagerman, 2003). Nonetheless, there exist only a few systematic examinations of *Fmr1* distribution from

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*Correspondence to: Justin R. Fallon, Department of Neuroscience, Brown University, Box 1953, 190 Thayer Street, Providence, RI 02912. E-mail: Justin_Fallon@brown.edu

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early embryonic to post-natal development (de Diego Otero et al., 2000). Although *Fmr1* expression pattern is broad, many studies have shown that *Fmr1* is transcribed in a tissue-specific manner and that its levels show dynamic changes during development. This expression pattern suggests that a complex interplay among *cis* and *trans* regulatory elements, along with epigenetic modifications underlie *Fmr1* gene expression during development.

Expression during embryonic development

In human embryos and fetuses, the brain and testis express high levels of *Fmr1* transcripts (Hinds et al., 1993). In 21–23 day old embryos, neural tube shows *Fmr1* expression (Agulhon et al., 1999; de Diego Otero et al., 2000), and entire brain regions (telencephalon, diencephalon, mesencephalon, metencephalon, and mylencephalon) show expression of *Fmr1* in 4–7 weeks old embryos, with especially high staining in the proliferating and migrating tissues (Abitbol et al., 1993; Agulhon et al., 1999). Moreover, non-neural structures such as the eye, cartilage, and gonads exhibit strong to moderate levels of *Fmr1* expression (Hanzlik et al., 1993; Agulhon et al., 1999; de Diego Otero et al., 2000). Similar findings have been reported in other species (Hinds et al., 1993; Bakker et al., 2000; de Diego Otero et al., 2000). In all of these studies, the expression of *Fmr1* and/or FMRP are the highest in cells or tissues derived from the ectodermal germ layer (e.g., pituitary, epidermis, spinal cord, neurons, hair follicle, adrenal medulla, and sensory cells), suggesting a possible involvement of *Fmr1* in ectodermal patterning. *Fmr1* expression has also been documented in the neural crest cells (NC; de Diego Otero et al., 2000) and branchial arches (Agulhon et al., 1999), which are of ectodermal origin. Expression of *Fmr1* in the NC is particularly interesting as many tissue structures affected in FXS are derivatives of NC. Clearly, there is precedence for studies that examine the regulation of *Fmr1* expression during early embryonic and neural development.

Expression during post-natal development

Similar to the embryonic expression patterns, both neural and non-neural tissues in the adult express *Fmr1*. In particular, the brain, testis, ovaries, thymus, esophagus, and spleen exhibit high levels of *Fmr1* transcripts (Hinds et al., 1993). In the central nervous system (CNS), olfactory bulb, hippocampus, and cerebellum exhibit high levels of *Fmr1* expression (Bakker et al., 2000; Lim et al., 2003). In neurons, *Fmr1* is localized to perikaryon and dendrites with some axonal staining (Feng et al., 1997). In recent years, there has been an intense investigation into the role of *Fmr1* expression in dendrites. However, its role in dendrites and synaptic plasticity has not been fully elucidated. Furthermore, it remains to be seen if FMRP confers different functions during embryonic and post-natal development.

REGULATION OF *Fmr1* TRANSCRIPTION

Recent studies have mapped the promoter of the *Fmr1* gene in various species and have identified several potential transcription factors associated with it (Drouin et al., 1997; Schwemmle et al., 1997; Carrillo et al., 1999). Furthermore, others have examined epigenetic changes such as the methylation status of CpG regions and CGG repeats in *Fmr1* promoter. The recent discovery that small interfering RNA (siRNA) can mediate transcriptional gene silencing has led to the proposal that siRNA may play a role in transcriptional silencing of *Fmr1* gene via epigenetic modification (Jin et al., 2004). While it remains to be determined whether such a mechanism exists, a growing interest in epigenetic regulation and chromatin remodeling process will undoubtedly reveal novel molecular pathways underlying the transcription of normal and mutated *Fmr1* alleles.

Genomic structure

The *Fmr1* gene is composed of 17 exons that span 38kb at Xq27.3 (Fig. 1; Eichler et al., 1993). Alternative splicing has been reported for the *Fmr1* gene in a variety of human and mouse tissues (Ashley et al., 1993; Verkerk et al., 1993; Verheij et al., 1995). Specifically, five regions of the *Fmr1* gene can be alternatively spliced to encode different protein isoforms known collectively as FMRP (Ashley et al., 1993; Verkerk et al., 1993). These splice isoforms show no known tissue specificity and their functional significance is presently unknown.

A handful of genes with known phenotypes are located within 7.5 million base pairs (bps) of the *Fmr1* gene. Interestingly, several biological phenotypes such as familial migraine, X-linked mental retardation (South African type), testicular germ cell tumor, and cornea dermoids are linked to regions near Xq27.3. Moreover, many nearby regions have been linked to or encode protein products that contribute to various forms of X-linked mental retardation (Fig. 2). Of particular interest is a folate-sensitive fragile site (FRAXE) that is located about 700,000 bps downstream of FMR1 site. A mutation at this site has been identified in several families whose members show a developmental delay without the additional molecular abnormalities associated with *Fmr1* in FXS. This site, now known as FMR2, was cloned and found to have a methylated GCC trinucleotide repeat sequence (Knight et al., 1993; Gecz et al., 1997).

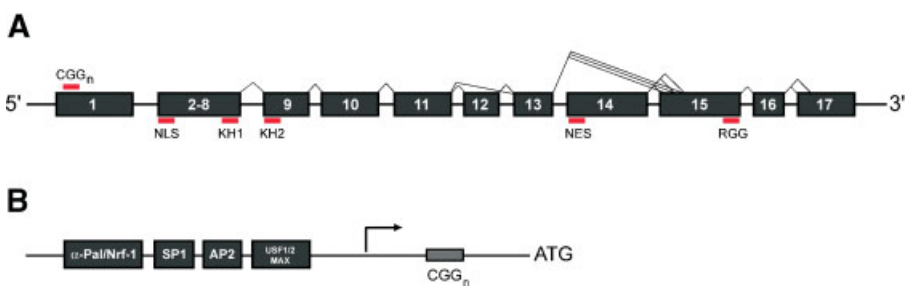

Fig. 1. Genomic and promoter structure of human *Fmr1*. **A**: The *Fmr1* gene consists of 17 exons that are alternatively spliced to encode several different protein isoforms. The protein product FMRP has several functional domains (red boxes) including three RNA-binding domains (KH1, KH2, and RGG box). It also has nuclear localization (NLS) and export signals (NES), allowing it to shuttle between the nucleus and cytoplasm. **B**: The promoter region contains several known transcription binding sites. The CGG repeat lies 149 bases downstream of the transcription start site (arrow).

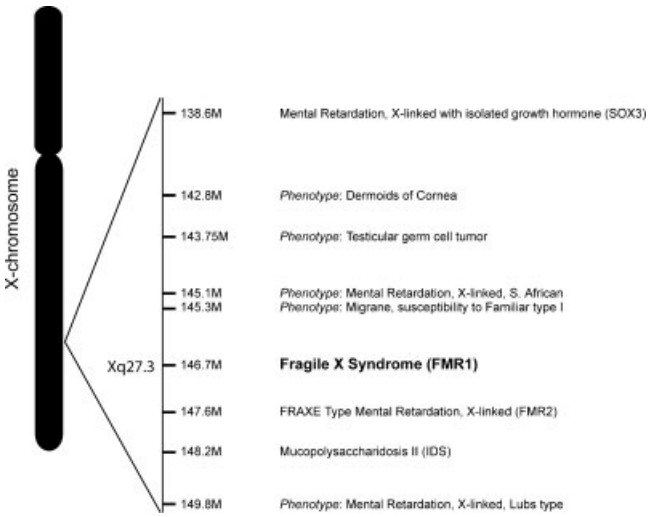

Fig. 2. Physical map of human X chromosome showing genes that are near *Fmr1*. Note that several genes implicated in mental retardation and testicular function lie within several million base pairs of *Fmr1*.

However, there is no evidence that FMR2 plays a role in FXS pathogenesis. Interestingly, in other trinucleotide repeat disorders, most notably the myotonic muscular dystrophy, CTG repeat expansion at the 3′UTR affects the expression of a nearby gene (Ranum and Day, 2004). A similar scenario might be hypothesized for the presence of CGG repeat expansion at the 5′UTR of *Fmr1*. However, due to the sparseness of genes near Xq27.3, it does not seem likely that the CGG repeats in *Fmr1* have significant influence on the expression of its neighboring genes.

Promoter structure

The *Fmr1* promoter is rich in CpG islands and lacks a canonical TATA box. A TATA-like sequence (TTACA) is present as well as an initiator-like element that was thought to be important in transcription initiation (Drouin et al., 1997). However, removal of either the TATA-like sequence or the initiator-like element does not abolish *Fmr1* gene activity (Kumari and Usdin, 2001), suggesting a novel, as yet unidentified mechanism for initiating *Fmr1* transcription. Nonetheless, a transcription initiation site (TIS) has been identified by primer extension studies to be located 134 bp upstream of the CGG repeats and 26 bp downstream of a TATA-like sequence (TTACA; Hwu et al., 1997). This site had been thought to be the predominant initiation site; however, recent work has shown that the transcription start site redistributes with CGG repeat expansion (Beilina et al., 2004). The mechanism by which downstream elements such as the CGG repeats alter transcription start sites is not known. Moreover, it is not known if cells utilize the additional start sites in normal *Fmr1* transcription. One study has shown that DNA CGG repeat-binding protein (CGGBP1 or p20) reduces the activity of *Fmr1* promoter (Muller-Hartmann et al., 2000), suggesting that both *cis* and *trans* elements influence the recruitment of RNA polymerase II to the proper initiation site.

Transcription factors

The earliest insights into how *Fmr1* transcription is modulated came from in vivo DMS (dimethylsulfate) footprinting and in vitro DNA binding analyses. Results from various cell lines have identified several transcription binding sites on the *Fmr1* promoter including SP1, AP-2α, α-Pal/Nrf1, and USF1/2 (Fig. 1; Drouin et al., 1997; Schwemmle et al., 1997; Carrillo et al., 1999; Kumari and Usdin, 2001). A promoter deletion analysis demonstrated that regions containing the above transcription binding sites are important for activating *Fmr1* transcription (Carrillo et al., 1999; Kumari and Usdin, 2002).

The *Fmr1* promoter also harbors several potential silencer elements, such as NF1 and Max (Carrillo et al., 1999). A recent study using chromatin immunoprecipitation assay has shown the binding of Max to the endogenous *Fmr1* promoter in HeLa cells (Smith et al., 2004), suggesting the importance of repressor elements in regulating *Fmr1* expression. USF1/2 has also been implicated in *Fmr1* transcription, however, conflicting data exist on whether it can function as an activator or a repressor (Kumari and Usdin, 2001; Smith et al., 2004).

The identification of these transcription factors has undoubtedly advanced our understanding of the regulatory components influencing *Fmr1* gene expression. Nevertheless, these results need to be interpreted in the context of the type of cell line or tissue used in the study. Indeed, many transcription factors, including those identified to be important in *Fmr1* transcription, such as AP-2α and α-Pal/Nrf1, display unique expression patterns at both the cellular and tissue level. Moreover, as the levels of *Fmr1* change during development, it is important to examine whether particular transcription factors exert selective influences at specific developmental stages. For instance, AP-2α is highly expressed only during embryonic and prenatal development and is thought to be essential for various aspects of neural crest and CNS development in embryos (Zhang et al., 1996; Feng and Williams, 2003). Thus, AP-2α might be a key regulator of *Fmr1* expression during embryonic development; in later stages other transcription factors are likely to play more prominent roles. Clearly, there is a need for functional studies to establish where, when and how these transcription factors regulate *Fmr1* expression.

Role of neuronal activity in *Fmr1* expression

Neural activity plays a profound role in gene expression, and the activity-responsive genes in turn play important roles in synaptic function (West et al., 2002). Recent studies suggest that neural activity can alter *Fmr1* expression in several neural systems. For example, electroconvulsive shock (ECS), which evokes seizure activity in the brain and induces immediate-early gene expression (Cole et al., 1990), upregulates *Fmr1* transcript levels in the dentate gyrus (Valentine et al., 2000). Moreover, physiological stimuli can also regulate Fmr1 mRNA levels. *Fmr1* transcript levels in the olfactory bulb increase after exposing rats to a novel odorant. This regulation is also bi-directional in that odor deprivation results in reduced *Fmr1* expression (Lim et al., 2003).

Neural experience also influences FMRP expression in several systems. Increase in FMRP expression has been observed in barrel cortex following whisker stimulation (Todd and Mack, 2000), and in the motor cortex of rats trained on motor-skill tasks (Irwin et al., 2000). Furthermore, exposing dark-reared rats to light induces the transient expression of FMRP in the visual cortex (Gabel et al., 2004). This expression peaks at 15–30 min and returns to baseline on the same time scale. Interestingly, *Fmr1* transcript levels do not change

in the barrel and visual cortices, suggesting a post-transcriptional regulation of *Fmr1* expression in these two systems, (Todd and Mack, 2000; Gabel et al., 2004). In the motor cortex study, transcript levels were not measured (Irwin et al., 2000). Collectively, these studies indicate that the regulation of *Fmr1* expression is highly modality-specific. The different neural systems can utilize either transcriptional or post-transcriptional mechanisms to modulate *Fmr1* or FMRP levels. Nonetheless, further study of all of these systems will reveal a multi-layered network underlying *Fmr1* regulation in each of them. More importantly, such studies will potentially provide novel insights into the molecular events underlying the heightened behavioral response to sensory stimuli in individuals with FXS (Miller et al., 1999; Hagerman and Hagerman, 2003).

Fmr1 transcription and potential signaling pathways

Past studies examining transcription factors have yielded clues to the signaling pathways underlying *Fmr1* expression. For instance, neural activity influences the expression of two transcription factors that are known to associate with the *Fmr1* promoter, AP-2α (Donaldson et al., 1995; Olsson et al., 1995; Li et al., 2000) and USF1/2 (Chen et al., 2003). In particular, USFs can modulate the expression of several activity-dependent genes via calcium-mediated signaling pathway (Chen et al., 2003). As described earlier, USFs associate with the *Fmr1* promoter, hence signaling pathways involving calcium might regulate *Fmr1* transcription. Another potential pathway involves AP-2α and SP1 (Luscher et al., 1989; Darrow et al., 1990; Wanner et al., 1996; Xie et al., 1998; Suzuki et al., 1999; Okumura et al., 2004), which are responsive to retinoic acid, suggesting a possible involvement of retinoid signaling pathway in regulating *Fmr1* expression.

A recent study showed that KCl-depolarization rapidly increased Fmr1 and FMRP trafficking to dendrites and synapses (Antar et al., 2004). While the increases in FMRP levels were not dependent on new protein-synthesis, it is not known if altered transcriptional activity underlies the increased dendritic localization of Fmr1 mRNA. The excitatory neurotransmitter glutamate is another molecule that could potentially regulate *Fmr1* expression. Indeed, both metabotropic glutamate (mGluR) and *N*-methyl D-aspartate (NMDA) receptors have been implicated in post-transcriptional regulation of *Fmr1* (Huang et al., 2002; Gabel et al., 2004; Weiler et al., 2004). However, none of these signaling pathways have yet been linked to regulating *Fmr1* transcription.

EPIGENETIC REGULATION OF *Fmr1*

In addition to transcription factors, recent studies have shown that epigenetic modifiers are equally important regulators of gene expression (Berger, 2002; Bird, 2002; Egger et al., 2004; Jiang et al., 2004). Epigenetic factors modify chromatin structure without altering its sequence. Cells utilize several different types of epigenetic mechanisms including methylation, acetylation, phosphorylation, ADP ribosylation, and ubiquitination to regulate gene expression. Although it has been known that these processes are important in basic biological processes such as X-inactivation (Vasques et al., 2002), only recently have we begun to appreciate their roles in many developmental and neurological diseases, including the FXS (Egger et al., 2004).

Several studies have examined the role of histone modifications in the *Fmr1* gene from fragile X patients (Coffee et al., 1999, 2002; Pietrobono et al., 2005). Two epigenetic marks, methylation of histone H3 at lysine 4 (H3-K4) and at lysine 9 (H3-K9) have been implicated to play an important role in determining the state of *Fmr1* gene activity. Furthermore, other epigenetic marks such as methylation of CpG sites on the *Fmr1* promoter become additional targets of regulation by *trans*-acting factors. For instance, a well-characterized ATPase-dependent chromatin remodeling complex, SWI/SNF together with methyl-CpG binding protein, MeCP2 directly associate with the hypermethylated regions of *Fmr1* gene to repress its transcription (Harikrishnan et al., 2005). Hence, there appears to be multiple levels of epigenetic regulation acting in parallel to define the state of *Fmr1* transcription.

Chromatin plasticity and the regulation of *Fmr1* transcription

Recent studies have recognized the dynamic nature of chromatin modification. The idea that methylation states are plastic and that it can be modified throughout the lifetime of an organism by a wide range of environmental factors is no longer a hypothesis. For instance, altering maternal nursing behavior has been linked to changes in the epigenome of their offspring (Weaver et al., 2004). Rat mothers who actively groom their pups show increased histone acetylation and decreased methylation of the glucocorticoid receptor gene promoter. Other changes in chromatin structures have been reported for the transcription factor, BDNF in cultured neurons. The studies showed that methylation of specific CpG sites in the BDNF promoter undergo activity-dependent modification (Tao et al., 2002; Chen et al., 2003; Martinowich et al., 2004). This remarkable phenomenon of activity-induced changes in the epigenome will play an important role in the study of learning and memory, and perhaps in the regulation of *Fmr1* transcription. As stated previously, experience can influence the transcriptional activity of *Fmr1*. Moreover, certain epigenetic changes are present in the *Fmr1* promoter, albeit in the mutated *Fmr1* allele (Coffee et al., 1999, 2002; Pietrobono et al., 2005). Therefore, it will be of great interest to determine if activity can mediate epigenetic changes in the *Fmr1* promoter to regulate the expression of *Fmr1*.

RNA INTERFERENCE AND THE REGULATION OF *Fmr1* TRANSCRIPTION

Two essential components of the 'RNA-induced silencing complex' (RISC), Argonaute2 (AGO2), and Dicer associate with the *Drosophila* homolog of FMRP (Caudy et al., 2002; Ishizuka et al., 2002; dFMR1). In addition, both micro-RNA (miRNA) and siRNAs co-immunoprecipitate with dFMR1 (Caudy et al., 2002; Ishizuka et al., 2002) as well as its mammalian homolog FMRP (Jin et al., 2004), further linking the idea that FMRP plays a role in the RNAi pathway. However, the loss of FMRP does not perturb the underlying RNAi or miRNA mediated gene silencing mechanism, suggesting that it is not a critical component of the RNAi pathway (Caudy et al., 2002; Ishizuka et al., 2002). Nonetheless, it has been hypothesized that FMRP, an RNA-binding protein, acts as a chaperone for recruiting specific siRNAs or miRNAs onto RISC (Siomi et al., 2004). Such a mechanism is particularly appealing in neurons where a tight temporal-spatial regulation of RNA localization

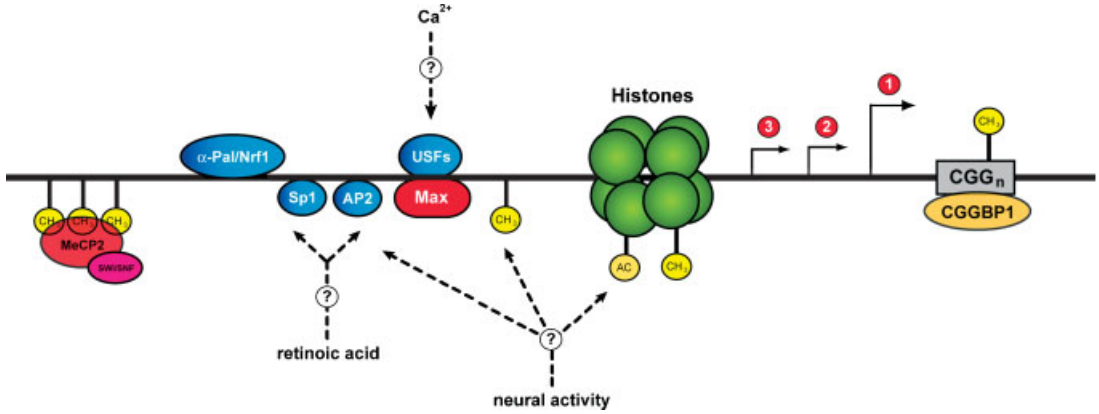

Fig. 3. Model for *Fmr1* transcription regulation. Transcription factors α-Pal/Nrf1, Sp1, AP-2α, and USFs are known to bind *Fmr1* promoter and activate its expression. Some of these factors might be influenced by higher level signaling cascades that involve retinoic acid, Ca^{2+}, and/or neural activity. Max is a newly identified repressor of *Fmr1* transcription. Epigenetic factors also play an important role in the regulating *Fmr1* expression. Acetylation and methylation of the N-terminal tail of histones as well as the methylation of CGG repeats and CpG sites on the promoter has been shown to modulate *Fmr1* activity. It is also known that CGG binding proteins (CGGBP1) can alter promoter activity. When *Fmr1* promoter becomes hypermethylated, a methyl-CpG binding protein, MeCP2 is recruited along with ATPase-dependent chromatin remodeling complex SWI/SNF to repress *Fmr1* gene transcription. Three different transcription start sites are shown (numbered 1–3). Upstream start sites are preferentially used with increasing CGG repeats.

and translation is crucial for proper neuronal maturation and function (Job and Eberwine, 2001).

It has been thought that cells utilize post-transcriptional or translational mechanisms to direct RNAi-mediated gene silencing. However, both siRNA and miRNA can also act at the level of transcription by associating with the 'RNA-induced initiator of transcriptional gene silencing' (RITS) complex (Noma et al., 2004; Verdel et al., 2004) to suppress the transcription of the targeted gene (Noma et al., 2004). Specifically, siRNAs target the promoter region of a gene and induce DNA methylation of the targeted sequence to silence gene expression (Chan et al., 2004; Kawasaki et al., 2004; Morris et al., 2004). Therefore, the link between epigenetics and siRNA mediated gene silencing is emerging as a novel way to regulate gene expression.

Interestingly, it has recently been proposed that RNAi-mediated methylation of the expanded CGG repeats in FXS might be the culprit behind the silencing of *Fmr1* transcription (Jin et al., 2004). In this model, siRNA-like molecules target the CGG repeats, accompanied by the recruitment the RITS complex, and de novo DNA methyltransferases (DMTases), which initiate local methylation of *Fmr1* gene. As described earlier, in the RNAi pathway that mediates post-transcriptional gene silencing, FMRP is thought to recruit specific siRNAs onto RISC. Perhaps, in a similar fashion FMRP may associate with the RITS complex to deliver siRNAs to their targets to induce epigenetic modifications. As many molecules involved in chromatin remodeling are RNA binding proteins (Jeffery and Nakielny, 2004), it is likely that FMRP itself might play a role in certain aspects of chromatin remodeling process.

FROM ANALYSIS OF *Fmr1* PROMOTER TO UNCOVERING FMRP FUNCTION

As described in the previous sections, new insights into the regulation of *Fmr1* transcription are beginning to define how we think about its potential functions. Our efforts to better understand the molecular basis of FXS in the past several years have delved into the intricate and complex nature of *Fmr1* gene regulation. The identification and characterization of transcription factors bound to the *Fmr1* promoter have hinted at several potential signaling pathways that may modulate *Fmr1* expression (Fig. 3). Indeed, the function of a protein has been often elucidated by a systematic analysis of the regulatory elements within the encoding gene (Werner, 2003). The utilization of such an analysis to the *Fmr1* promoter will undoubtedly aid in unmasking FMRP's function, and more importantly, it will shed new light on FXS.

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