# These CGGs Are Making Me Fragile, Mentally That Is...

**Student group names kept anoynymous**
Department of Biology, Lake Forest College, Lake Forest, IL 60045, USA

**Fragile X Syndrome is one of the most common forms of inherited mental retardation. Symptoms include learning disabilities, facial abnormalities, and social instability. The disease is caused by the expansion and hypermethylation of the CGG triplet in the fragile X mental retardation gene 1 (FMR1), leading to a loss of production of the fragile X mental retardation protein (FMRP), which is an RNA-binding protein. The mechanism of expansion and methylation of the CGG triplet repeat, as well as the roles of FMRP have yet to be identified. Current research has shown that expansion of triplet repeats might be due to polymerase pausing during DNA replication as a result of formation of stable DNA secondary structures, such as hairpin loops and tetrahelices. Hypermethylation has been found to repress transcription either by recruiting histone deacetylase or by blocking transcription factor binding. Researchers have also found several methods for testing which RNAs are likely targeted by FMRP *in vivo*. These methods include mouse and fly models, microarray identification, and in vitro tests. There is no known cure for Fragile X syndrome but further research may help lead to treatments that can correct the FMR1 gene or replace the function of FMRP.**

**The Fragile Basics**

The fragile X syndrome is a sex-linked, mental retardation disease caused by a CGG trinucleotide expansion of the FMR1 gene. The gene is located on the X chromosome, at the site Xq27.3, and was found in 1991 after a great deal of earlier work pointed to the vicinity of the fragile X site as an explanation for the increased number of males with mental retardation (1). The site is called “fragile” because a piece of the chromosome appears as though it is separated, though it is not completely broken off (2).

**Fragile Characteristics**

Fragile X leads to abnormal physical characteristics including a long face, large ears, high arched forehead, hyper extensible joints, flat feet, velvet-like skin and enlarged testicles. Babies with fragile X have also been found to have higher than normal birth weights as well as problems with ear and eye development (1). Mental disabilities include schizophrenia or autism-like symptoms such as impaired verbal and nonverbal communication, weakened social interactions, and restricted and repetitive patterns of behavior. Males tend to exhibit the autism-like symptoms more often than females whereas females more often suffer from schizophrenia and extreme shyness, anxiety, and mood problems. When children who have fragile X grow older, they also display hypersensitivity, hyperactivity, aggression and excessive social anxiety (3). Individuals who suffer from fragile X usually don’t know they have it since the disease’s symptoms are similar to those of other mental diseases. Thus, the only way to know if an individual has fragile X is through DNA testing (2).

**Fragile Population**

Fragile X is one of the most common causes of mental retardation. Because it is X-linked, it is more prevalent in females than in males due to random X chromosome heterochromatinization (3). In fact, approximately 1 in 4500 males are affected and approximately 1 in 9000 females are affected. On the other hand, females are two and a half times more likely than males to be carriers of the disease. There are three levels at which the CGG repeat can exist. A normal fragile X exon

contains 30 CGG repeats, whereas a premutated exon has between 60 and 200 repeats, and the fully mutated exon has over 230 repeats. People who have the premutated exon repeats are considered carriers, while people with the fully mutated exon suffer from fragile X symptoms (1).

**The Fragile Abnormalities on a Cellular Level**

The Fragile X syndrome is caused by mutations in the FMR1 gene such as deletions, point mutations, and most often expansions. All of these mutations result in the loss of FMRP production (4). The absence of FMRP is thought to cause abnormalities occurring in the development of dendritic spines in fragile X patients. The spines are often long, thin, and winding, preventing appropriate synaptic connections between neurons therefore inhibiting their message relays necessary for normal function. (4,5).

**Picking up the Shattered Pieces**

At this time there is no cure for fragile X syndrome, but with education and medication, the symptoms can be controlled. There are three major areas that are being studied at this time to help treat patients with fragile X.

The first procedure involves gene repair. This method would involve reactivating the FMR1 gene, through demethylation and histone deacetylase inhibition. This method looks promising because it is using the genes already present so that no new genetic material would have to be introduced (4).

The second procedure is gene therapy. This method involves inserting normal genes into the brain cells in order to correct the disease. This treatment has not been tried in humans, but insertion of healthy copies of the FMR1 gene into neurons of knockout mice has been successful (4).

The last treatment that is widely used today is psychopharmacology. Medications can help relieve patients of their fragile X symptoms. These medications have not been thoroughly tested, but once they are found to be effective, they will be used more readily than gene repair and gene therapy (4).

**Fragile Biology**

The FMR1 gene itself consists of 17 exons, and the CGG trinucleotide repeat is located within the first exon, at the 5'untranslated region (5'-UTR). In normal individuals, the CGG repeats are interrupted by single AGG sequences. It has been suggested that the loss of AGG interruptions can cause the CGG repeats to become unstable. When the CGG repeats reach full mutation, the gene becomes hypermethylated, the methyl groups attaching to the cytosine residues in the repeat. (1,6). Together, expansion and methylation result in silencing of the FMR1 gene (Figure 1) yet, scientists still don't know what the mechanism of this expansion and methylation are.

Once the FMR1 gene is silenced FMRP

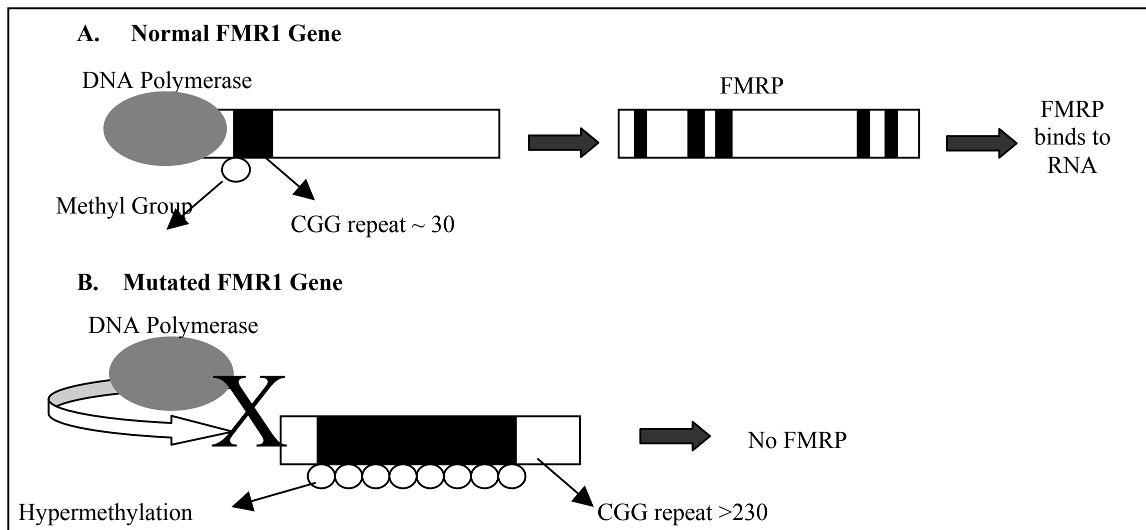


**Figure 1: Activities of the FMR1 Gene and FMRP in Normal and Fragile X cells.**
**A.** A normal FMR1 gene is transcribed and translated into FMRP.
**B.** DNA Polymerase cannot bind to FMR1 due to methylation and expansion. No FMRP is made.

production is inhibited. FMRP is an RNA binding protein that includes two KH domains and a RGG box. KH domains contain approximately 40-60 amino acids, many of which are hydrophobic. RGG boxes are 20-25 amino acids long with repetitions of arginine and glycine residues. FMRP is also known to contain a nuclear localization signal (NLS) and a nuclear export signal (NES), which help the protein enter and exit the nucleus (1).

Although the function of FMRP is still not established, the structural features of FMRP have lead to the development of one strongly supported model. This model suggests that FMRP shuttles specific mRNAs out of the nucleus and regulates translation of those mRNAs. FMRP enters the nucleus using its NLS, and when in the nucleus, associates with other proteins and mRNA to form a ribonucleotide particle (RNP). As FMRP uses its NES to exit the nucleus, the bound mRNA is also transported out of the nucleus. The subsets of proteins that form the RNP disassemble, but FMRP remains bound to mRNA and shuttles it along the dendrite or to a post-synaptic site in neurons. In this way, protein synthesis is localized in the required subcellular regions. FMRP may also be responsible for carrying the mRNA to polyribosomes. Translation, when required, takes places once the FMRP disassociates from the mRNA. The mRNAs that FMRP binds to could be important in neuronal development, and in the absence of FMRP the synthesis of these proteins could be deregulated (1,6). However, which RNAs FMRP targets, as well as the specific location of FMRP that binds to RNA, has yet to be determined.

**Look At Those CGGs Go**

Although the mechanism of FMR1 gene expansion still remains a mystery, a great deal of progress has recently been made. One proposal is that dynamic mutation of the FMR1 gene could involve a gene conversion event. This consists of replication of part of the CGG repeat from one allele and insertion of that copy into the other allele (7). Another hypothesis, the replication-based model, suggests that slippage of DNA during replication of the lagging strand causes CGG expansion. If long CGG repeats form stable secondary structures on the newly synthesized DNA strand, the strand will be inclined to slip during replication. The unusual structure would cause the DNA polymerase to pause, and the primer would have to relocate itself on the nascent strand to begin replication again (1,3,6,8). DNA repair enzymes could either edit out the extra bases or they could incorporate those bases into the newly synthesized strand. If the latter were the case, then the result would be an expanded gene (7).

Evidence for the replication-based model has been accumulating. Triplet repeats are hypothesized to form four types of unusual DNA structures: toroids, slipped structures, hairpin loops, or tetrahelices. Toroids and slipped structures, duplex DNA conformations, have been identified in expanded triplet repeats (9,10). In the case of fragile X, CGG triplets have been shown to form hairpin loops and tetrahelices.

**CGGs and Tetrahelices**

A study conducted by Fry and Loeb demonstrated the formation of tetrahelical structures in CGG repeats. An electrophorectic mobility test was done to determine what sizes of CGG repeats can form and stabilize the tetrahelical structure and also to test whether methylation affects the migration of CGG oligomers. The results indicated that migration of CGG oligomers was slower under nondenaturing conditions, when secondary structures could form, rather than denaturing conditions. Also, the longer and more methylated the CGG oligomers were, the slower their mobility. Next, the scientists tested the effects of different salt concentrations, incubation time, and DNA concentrations on formation of the secondary structure. Increased salt concentrations, increased time, and increased DNA concentrations were all positively correlated with the formation of the tetrahelical structure. Since the kinetics for the formation of this structure was shown to be second-order, this implied that the structure was an interstrand complex rather than monomolecular. Furthermore, when two different sized CGG oligomers were run together, there was formation of intermediate-size complexes. Thus the scientists concluded that the structure being formed was tetrahelical, and CGG repeats were capable of folding into these structures *in vitro* (11).

**CGGs and Hairpin Loops**

Nadel et al. have found evidence that CGG repeats fold into unimolecular hairpin loop structures. Electrophoretic mobility test was done under denaturing and nondenaturing conditions to determine whether CGG repeats form folded structures. The results displayed

that CGG oligomers move faster on the polyacrylamide gel under nondenaturing conditions as compared to denaturing conditions. Since greater mobility is characteristic of hairpin loop structures, it can be said that CGG repeats are forming similar structures. Another experiment revealed that the structure that CGG repeats form unfolds at higher temperatures. Under UV light, as temperature increased, the CGG oligomers were seen to absorb more UV-radiation. This result indicates that the secondary structure must be unfolding, exposing more bases, which in turn absorb more UV radiation (12).

In order to prove that it was the hairpin loop that CGGs were forming, clusters of thymidine residues were inserted into various CGG repeat sequences. Since thymines are known to form thymine-thymine dimers under UV light, the CGG sequences were run on polyacrylamide gel in the presence of UV light. The results demonstrated that there was a rapidly migrating species of covalently bonded or cross-linked DNA. This proved that in order for the thymine-thymine dimer to form, the CGG oligomer must have had to fold and form a hairpin loop (12). The folding of CGG repeats into these hairpin loop structures may cause slippage, delay DNA replication and contribute to expansion.

**CGGs Confuse DNA Polymerase**

Along with formation of secondary structures, pausing in DNA synthesis has also been shown in triplet repeats. Kang et al. have used DNA sequencing and primer extension to test whether DNA polymerase pauses in expanded CGG repeat sequences. It was found that pausing occurred at two sites, one near the third CGG repeat and one between the twenty-ninth and thirty-first CGG repeat in a 160 CGG repeat-long sequence (13). This result supports the hypothesis that the pausing and slippage of DNA can lead to expansion of CGG repeats (Figure 2). However, the direct relationship between formation of secondary structures and DNA pausing and slippage during replication still needs to be shown.

**Mechanism of Methylation**

In addition to the mechanism of expansion, the mechanism of methylation also had to be understood since the two were found to be related. Wang and Griffith used nucleosome reconstitution as well as gel shift assays to find the way in which expansion affects nucleosome assembly and subsequent methylation. Their results revealed that repeated CGG sequences make chromatin loose. This change in the chromatin's structure increases the DNA's accessibility to DNA methyltransferase, which places methyl groups on the cytosines of the repeated sequence. If methyltransferase has access to the expanded DNA it will hypermethylate it and thus inhibit transcription. Wang and Griffith also discovered that highly methylated DNA containing the repeat was half as efficient in assembling into nucleosomes as the unmethylated DNA of the same fragment length. (14)

**Histone Deacetylation**

Another way to inhibit transcription is by histone deacetylation. In deacetylation, the histone affinity for DNA increases thus condensing the chromatin and repressing gene expression (15). Scientists wondered whether this deacetylation was involved in fragile X since methyl cytosine-binding proteins (MeCP2) also bind to histone deacetylases. Coffee et al. did an experiment in which levels of histone acetylation between normal and fragile X syndrome cells were compared using chromatin immunoprecipitation. Results showed normal FMR1 cells having low levels of deacetylation whereas fragile X syndrome cells had high levels of deacetylation. One explanation for this result is that histone deacetylation can cause inhibited transcription in people who have fragile X (16).

**A Linking Model**

Since researchers knew that people with fragile X had high levels of both methylation and histone deacetylation, the following model was developed. Methyltransferase hypermethylates a repeating CGG sequence. Next, MeCP2 forms a complex with a transcription repressor, Sin3, which binds the hypermethylated CGG sequence to histone deacetylase (17). Histone deacetylase then deacetylizes the histones H3 and H4 of chromatin surrounded by the CGG repeat (Figure 2). This in turn leads to chromatin condensation and repressed transcription. Hence, methylation controls the level of histone acetylation and chromatin condensation (6,18,19).

**Another Relationship**

Scientists began to wonder how it came to be that active genes were not methylated. Using chromatin immunoprecipitation, Czervoni and Szyf found that the state of DNA methylation was not fixed and that demethylase

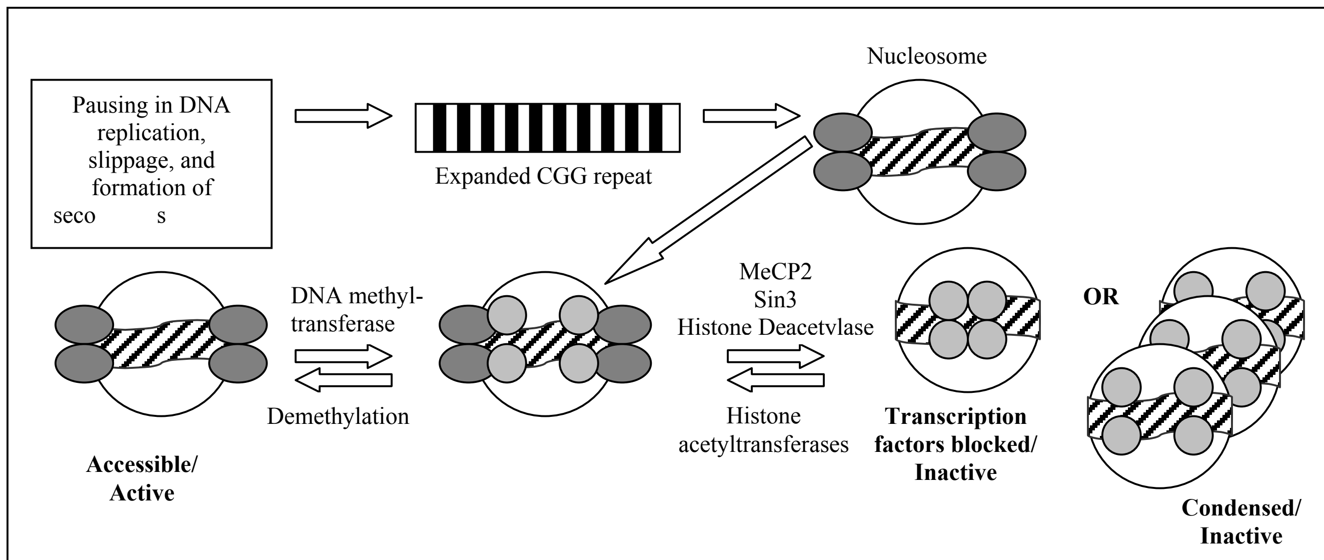


**Figure 2: The effects of expansion, methylation, and histone deacetylation on the FMR1 gene.**
Secondary structures in CGG repeats cause pausing in DNA replication and slippage in DNA. The result is an expanded CGG repeat, which loosely wraps around the nucleosomes that have acetyl groups (dark grey) already attached. Methyl transferase brings in methyl groups (light grey). These methyl groups can be demethylated and lead to possible transcription. The methyl- binding protein MeCP2 will bind to the hypermethylated repeats and form a complex with histone deacetylases via Sin3 (repressor protein). This can lead to blocking of the transcription factors or condensation of the chromatin.

were linked to elevated FMR1 expression. Also, when preventing α-Pal/Nrf-1 from binding, USF1 and USF2 bound less effectively (21). This led to the idea that there might be certain areas of the promoter where methylation causes more disruption transcription that others. Researchers found that when the methylated area was next to the promoter region, transcription was repressed. When the methylated area was moved 2.7 kilobases downstream from the promoter, transcription was unaffected. This showed that methylation does not always lead to transcriptional silencing but rather only methylation of certain areas of the gene silence it (22).

### Non Transcription Problems

A number of patients who have the premutation have been found to have normal or even above normal levels of FMR1 mRNA. This means that transcription did occur in these individuals and that the problem is elsewhere. Perhaps the increase in mRNA leads to a decrease in FMRP or gene expression is inhibited due to post-transcriptional problems such as translation. This area has much more research to be done (23,24,25).

### Approaching the Fragile Targets

Determining which RNAs target FMRP is very crucial to understanding fragile X. If the scientist could figure out which RNAs are quartets using *in vitro* techniques. Within test tubes they deleted or mutated particular segments of the RNA in order to find which pieces of the RNA were vital to binding with FMRP. They found that RGG boxes were more important than KH domains in order for binding to occur. Through the scientists' experimentation they found that there are 14 mRNAs that could potentially be targets for FMRP binding (25).

### The Chip to the Future

In another study by Victoria Brown, et al., they used a different approach, microarray identification, to figure out which mRNAs bound to FMRP. They used microarray identification by coimmunoprecipitating mRNA with FMRP. When the scientists added normal mRNAs to the wells of the microarray chip containing FMRP, 432 mRNAs were identified that had bound. When they took mRNAs from polyribosomes of fragile X cells, 251 mRNAs had abnormal profiles. This last result shows that without FMRP something strange occurs causing abnormalities to transpire. Between the two studies almost 70 percent of the mRNAs contained a G quartet, which corresponds with the *in vitro* findings. Through microarray identification they found evidence that mRNAs that usually bind to FMRP are dysregulated, fragile X may occur (27).

**Up, Up, and Away**

In another interesting experiment scientists used *Drosophila* models to experiment with RNA targets. The scientists came up with a fly fragile X syndrome model that used loss-of-function mutants and also overexpression of the FMR1 homolog, (dFXR). In this study they found that the dFXR binds to Futsch, which is a strand of RNA. When dFXR was not present, an overproduction of Futsch was made, resulting in symptoms resembling fragile X. When there was an overproduction of dFXR not enough Futsch was made, also causing fragile X-like symptoms. So, it seems that dFXR inversely regulates Futsch expression (28).

The dFXR also plays a role in synaptic terminal growth. When the dFXR was not present the synaptic terminal was much longer than expected. When the dFXR was overproduced the synaptic terminal was much shorter (28).

So, these results indicate that the dFXR binds to and regulates the translation of Futsch and ultimately plays a role in synaptic growth and function.

In a review article by Michael D. Kaytor and Harry T. Orr debated whether these results would also be seen in mammals. There is a mammalian homolog to Futsch, the microtubule associated protein MAP1B. This protein was identified as a target of murine FMRP and human FMRP in the microarray and the *in vitro* studies. The results in the *Drosophila* study suggest that synaptic alterations seen in patients with fragile X to be caused by the uncontrolled regulation of MAP1B expression centered at the synapse (29).

**Down For the Count**

Knockout mice are one of the more widely used *in vivo* methods. In a study conducted by Thomas A. Comery et al., the FMR1 gene was removed from mice. As a result, these mice lacked expression of FMRP. This lack of expression led to abnormal dendritic spines in the fragile X knockout mice. Their spines were longer, thinner, and more winding than those of the normal mice (5). The abnormal mice spines can be compared to the postsynaptic spines of fragile X patients. Furthermore, the spine density of the knockout mice was greater, which could mirror impaired developmental organizational processes of synapse elimination and stabilization as well as pruning (5). Therefore, these dendritic spine abnormalities suggest that the lack of FMR1 gene, which leads to the lack of FMRP is one of the main causes of these abnormalities, which supports studies conducted by Darnell, et al, Brown, et al., and Zhang et al.

**Seeking a Stable Future**

Studies have shown that CGG repeats are capable of forming secondary structures such as hairpin loops and tetrahelical structures. The fact that DNA polymerase pauses during when replicating CGG repeats has also been demonstrated. However, whether CGG repeats for secondary structures in vivo and whether this causes pausing in DNA synthesis in a fragile X patient, still needs to be shown. In addition, though the replication-based model explains how slippage of DNA can occur and result in expansion of the gene, it does not explain how a massive expansion can be generated during transmission from premutation to full mutation. The exact timing of replication is also unknown.

Studies have found that drugs such as 5-azadeoxycytidine can be used in vitro to demethylate DNA. This would then allow for transcription to occur. If we could find a way to use the drug in vivo without affecting the methylation of other DNA maybe we could restore the mutant FMR1 gene (2).

Three drugs have also been found to induce histone hyperacetylation. This could lead to the restoration of fully mutated FMR1 if combined with drugs that demethylate (31).

*In vitro* techniques have been found to be very useful in my different aspects of figuring out the cause of fragile X syndrome, especially with determining the exact RNAs that target FMRP. Scientists have found ways to begin studying this area *in vivo*, but it is still unknown if the MAP1B RNA is as crucial in humans as in other mammals. If it is found that the MAP1B is crucial in humans, a cure could be devised.
In the *Drosophila* study they only examined the Futsch mRNA, so the exact number of fly mRNAs that are regulated by dFXR is unknown. More testing would need to be done to see if other crucial mRNAs are present within the fly, which could be used to explain previous studies and lead the search for future studies dealing with the RNA targets of FMRP (29).

**Conclusion**

The field of Fragile X research has come a long way since its discovery in 1991. The mystery of how the syndrome works is being solved yet there is still much more to be

understood about the molecular basis of the disease. Advances in Fragile X research will help scientists comprehend other triplet diseases as well as neurodegenerative diseases.

**Acknowledgements**

We would like to thank Dr. DebBurman for all of his patience and guidance as well as Jennifer McGuire for her writing expertise.

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