SUPPLEMENTAL MATERIAL

CGG-repeat constructs

pSP6-FMR1 5′UTR(n CGG)-FL-polyA - An ampicillin-selectable *FMR1*-FL plasmid with 99 CGGs [pSP6-*FMR1*(99 CGG)-FL-polyA], previously described[1] has an SP6 promoter, *FMR1* 5′UTR beginning at *FMR1* Inr I, firefly luciferase (FL) reporter, and polyA tail. The plasmid also has a reverse-orientation, low-copy bacterial origin (i.e., opposite directions of replication fork migration and reporter gene transcription), which improves CGG-repeat stability during cloning. To change the number of CGG repeats, the plasmid is digested with BlpI and XhoI (all restriction enzymes from New England Biolabs, Inc, Ipswich, MA; NEB), which lie within the *FMR1* 5′UTR, one on either side of the CGG repeat, thereby removing the repeats and much of the 5′UTR. The larger fragment of this digestion was then ligated to the smaller, *FMR1* 5′UTR/CGG-repeat fragments of similarly digested plasmids[2] that contain 0, 16, 30, or 62 CGG repeats. Thus, a series of *FMR1* 5′UTR plasmids, designated *FMR1* 5′UTR(n CGG)-FL, was assembled with the indicated number of CGG repeats.

pSP6-Synthetic 5′UTR(n CGG)-FL-polyA - The *FMR1* 5′UTR was removed from pSP6-*FMR1* 5′UTR(n CGG)-FL-polyA and replaced with the following 82-base 5′UTR: 5′-GAATACAAGCTTGGGCTGCAGGTCGACCAAACATTAGATATCCATCGATACTCGAGCTTAG GGTACCGTTCAGATAGCCACC-3′, creating synthetic 5′UTR(0 CGG)-FL. CGG repeats were inserted into this plasmid as follows: synthetic 5′UTR(0 CGG)-FL was digested with EcoRV and XhoI. *FMR1* 5′UTR(CGG)-FL plasmids with 16, 30, 62, or 99 CGGs were digested with BlpI and XhoI, the small fragment purified from the rest of the plasmid by QIAquick gel extraction (Qiagen, Inc., Valencia, CA) and then digested with BstUI, yielding fragments that contain the CGG repeat plus 7 nt on the 3′ end. These CGG-repeat fragments were ligated into the EcoRV/XhoI-digested synthetic 5′UTR(0 CGG)-FL plasmid, creating a series of CGG-repeat constructs [synthetic 5′UTR(n CGG)-FL] in the synthetic 5′UTR context. The CGG repeat begins

at base 40 of the synthetic 5′UTR.

Hairpin plasmids

FMR1 5'UTR(HP)-FL - *FMR1*5′UTR(0 CGG)-FL was digested with PvuII and XhoI, which removes the CGG repeat by digesting on either side of it. The following sequence was inserted, which includes the HP (italicized) and restores the surrounding bases: 5′-CG*GGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCC*CTGGCC-3′

FMR1 5'UTR(HP/GAG)-FL - The same cloning strategy was used as for the *FMR1*5′UTR(HP)-FL construct, except that the insert was 5′-CG*GGGGCGCGAGGAGGCGGCTGCAGCCGCCTCCTCGCGCCCC*CTGGCC-3′. Sequence differences between HP and HP/GAG are underlined.

Synthetic 5'UTR(HP)-FL - Synthetic 5′UTR(0 CGG) was digested with EcoRV and XhoI, whose sites lie in the middle of the 5′UTR, and the following sequence, which includes the HP and restores the surrounding sequence removed during digestion, was inserted: 5′-A*GGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCC*TCCATCGATAC-3′. The 40-base HP, indicated by italics, begins 41 bases from the 5′ end of the resulting 5′UTR.

Synthetic 5'UTR(HP/GUG)[no stop]-FL - The stop codon (TAG) that lies within the synthetic 5′UTR, between the HP and the AUG start codon, was changed to TTA by digesting synthetic 5′UTR(HP)-FL with XhoI and NcoI, which both lie downstream of the HP and upstream of the AUG start codon. The following sequence was then inserted 5′-TCGAGCTTTAGGTACCGTTCAGATAGCCAC-3′, which changes the TAG (stop) to TTA (leucine) while maintaining the surrounding 5′UTR sequence.

5′HP-FMR15'UTR(30 CGG)-FL - *FMR1*5′UTR(30 CGG)-FL was digested with NaeI, upstream of the SP6 promoter, and BlpI, which cuts 10 bases into the 5′ end of the 5′UTR. The following sequence 5′-GGCATTTAGGTGACACTATA**G**ATCAGGCGCTC*GGGGCGCGTGGTGGCGGCTGCAGCCGC*

CACCACGCGCCCC-3′ was inserted into the vector portion of this digestion. The underlined region is the SP6 promoter. *FMR1* transcription begins at the second-to-last underlined base (bold G) of the SP6 promoter. The HP (italicized) begins 13 bases from the 5′ end of the transcript.

5''HP-FMR15'UTR(30 CGG)-FL - The following sequence, including HP, was added to the 5′ end of *FMR1*5′UTR(30 CGG)-FL: 5′-GAACATTTGTA*GGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCC*-3′. The HP sequence is italicized, and begins 12 bases from the (new) 5′ end of the message.

5'HP-Synthetic 5'UTR(30 CGG)-FL - Synthetic 5′UTR(30 CGG)-FL was digested with HindIII and PstI, which cut 11 and 16 bases from the 5′ end of the 5′UTR, respectively. The following sequence was inserted: 5′-AGCTT*GGGGCGCGTGGTGGCGGCTGCAGCCGCCACCACGCGCCCC*GGGCTGCA-3′. The insert restores the synthetic UTR sequence and HP (italics) begins at base 13 of the 5′UTR.

AUG knockout

Synthetic 5'UTR(0 CGG)-FL[-AUG] - Using the Quikchange II site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol, the first and second AUGs were changed to TAG and ATC, respectively. The primers used were 5′-CCGTTCAGATAGCCACCTAGGAAGACGCCAAAAAC-3′, 5′-GGTTTTGGCGTCTTCCTAGGTGGCTATCTGAACGG-3′ to change the first AUG; 5′-CTATCCGCTGGAAGATCTAACCGCTGGAGAGCAAC-3′ and 5′-GTTGCTCTCCAGCGGTTAGATCTTCCAGCGGATAG-3′ to change the second AUG.

FMR1 transcription start site III (Inr III)

The following sequence was added to the 5′ ends of *FMR1*5′UTR(n CGG)-FL with 0, 16,

30, 62, and 99 CGG: 5′-

GAGTGACGTGGTTTCAGTGTTTACACCCGCAGCGGGCCGGGGGTTCGGCCTC-3′. The first two, underlined bases of the *FMR1* 5′UTR were changed from AC to GA, in order to preserve the SP6 promoter. These constructs are designated *FMR1(InrIII) 5'UTR(n CGG)-FL*.

pRL-CMV

The *Renilla* luciferase (RL) plasmid pRL-CMV was obtained commercially (Promega Corp, Madison, WI).

References

1. Khateb, S., Weisman-Shomer, P., Hershco-Shani, I., Ludwig, A. L. & Fry, M. (2007). The tetraplex (CGG)n destabilizing proteins hnRNP A2 and CBF-A enhance the *in vivo* translation of fragile X premutation mRNA. *Nucleic Acids Res.* **35**, 5775-88.
2. Chen, L. S., Tassone, F., Sahota, P. & Hagerman, P. J. (2003). The (CGG)n repeat element within the 5' untranslated region of the *FMR1* message provides both positive and negative *cis* effects on *in vivo* translation of a downstream reporter. *Hum. Mol. Genet.* **12**, 3067-74.