

The quadruplex r(CGG)_n destabilizing cationic porphyrin TMPyP4 cooperates with hnRNPs to increase the translation efficiency of fragile X premutation mRNA

Noa Ofer, Pnina Weisman-Shomer, Jeny Shklover and Michael Fry*

Department of Biochemistry, Rappaport Faculty of Medicine, Technion—Israel Institute of Technology, Haifa 31096, Israel

Received November 18, 2008; Revised February 15, 2009; Accepted February 16, 2009

ABSTRACT

The 5' untranslated region of the *FMR1* gene which normally includes 4–55 d(CGG) repeats expands to > 55–200 repeats in carriers of fragile X syndrome premutation. Although the levels of premutation *FMR1* mRNA in carrier cells are 5–10-fold higher than normal, the amount of the product FMR protein is unchanged or reduced. We demonstrated previously that premutation r(CGG)_n tracts formed quadruplex structures that impeded translation and lowered the efficiency of protein synthesis. Normal translation could be restored *in vivo* by the quadruplex r(CGG)_n destabilizing action of CBF-A and hnRNP A2 proteins. Here we report that the quadruplex-interacting cationic porphyrin TMPyP4 by itself and in cooperation with CBF-A or hnRNP A2 also unfolded quadruplex r(CGG)_n and increased the efficiency of translation of 5'-(CGG)₉₉ containing reporter firefly (FL) mRNA. TMPyP4 destabilized *in vitro* a (CGG)₃₃ intramolecular quadruplex structure and enhanced the translation of 5'-(CGG)₉₉-FL mRNA in a rabbit reticulocyte lysate and in HEK293 cells. The efficiency of translation of (CGG)₉₉-FL mRNA was additively increased in cells exposed to TMPyP4 together with CBF-A. Whereas low doses of TMPyP4, CBF-A or hnRNP A2 by themselves did not affect the *in vivo* utilization of (CGG)₉₉-FL mRNA, introduction of TMPyP4 together with either protein synergistically augmented its translation efficiency.

INTRODUCTION

The 5' untranslated region (UTR) of the first exon of the *FMR1* gene includes in normal humans 4–55 repeats of a

d(CGG) trinucleotide. Dynamic mutation that expands the d(CGG)_n sequence to > 200–2000 repeats sets off fragile X syndrome (FXS), the most common cause of inherited mental retardation (1,2). Following its massive expansion, the repeat tract and an adjacent CpG island become hypermethylated and associated histones are modified such that *FMR1* is silenced and its FMRP product protein is not synthesized (3). While not developing FXS, carriers of *FMR1* premutation alleles that have > 55–200 d(CGG) repeats do present diverse clinical involvements (4,5). Most prominently, about 20% of the premutation female carriers exhibit premature ovarian failure (POF) (4,6) and up to one-third of the male carriers of a premutation allele present fragile X associated tremor-ataxia syndrome (FXTAS) (7–9).

Notably, in contrast to the absence of *FMR1* transcripts in FXS cells, peripheral blood leukocytes that have (CGG)_{100–200} premutation repeats produce 5–10-fold higher amounts of *FMR1* mRNA than cells of normal human subjects (10–12). A similar accumulation of excess mRNA was also observed in a mouse premutation model (13). Yet, despite the excessive amounts of *FMR1* transcripts, levels of their product protein FMRP remain unchanged or are lower than normal in cells of premutation carriers (11,14). The apparent reduced efficiency of translation of *FMR1* mRNA is consistent with a reported diminished association of premutation *FMR1* mRNA with polysomes in lymphoblastoid cell lines (14). Similarly, despite their normal steady-state level of *FMR1* mRNA, cells of a mildly affected FXS individual with *FMR1* alleles that included more than 200 (CGG) repeats displayed markedly reduced FMRP synthesis. Moreover, the *FMR1* mRNA molecules in these cells were shown to be associated with stalled 40S ribosomal subunits (15). Put together, the gathered evidence suggested that hindered polysome formation and stalled ribosome progression along premutation *FMR1* mRNA result in decreased efficiency of its translation *in vivo*. A likely source of the

*To whom correspondence should be addressed. Tel: +972 4 829 5328; Fax: +972 4 851 0735; Email: mickey@tx.technion.ac.il

inefficient protein synthesis is the folding of the 5'-UTR (CGG)_n premutation RNA tract into secondary structures. Such structures may well hamper the formation of polysomes and diminish the productive migration of ribosomes along the mRNA. Indeed, (CGG) repeat sequences in RNA were shown to readily fold into hairpin structures (16,17) and to form intra- and intermolecular tetraplexes (18,19). Translation has been shown to be impeded by quadruplex structures in diverse mRNA molecules (20–22). More pertinently, we reported that premutation (CGG) repeats placed in the 5' UTR region of a firefly luciferase (FL) reporter gene fold in FL mRNA transcripts into quadruplex structures that decreased the efficiency of their translation *in vitro* and *in vivo*. Moreover, two member proteins of the hnRNP family, CBF-A and hnRNP A2, that destabilize quadruplex (CGG)_n were demonstrated to alleviate in living cells the impediment to translation (19). Overall, these results strongly suggested that by folding into tetraplex structures, the 5' UTR premutation (CGG)_n sequence in mRNA obstructs translation and that the resolution of the tetrahelical secondary structure can restore protein synthesis to its normal level.

Reinstating normal efficiency of *FMRI* mRNA translation in FX carrier cells can conceivably assuage some of the clinical sequelae of the *FMRI* premutation. However, exogenously introduced proteins such as CBF-A or hnRNP A2 that resolve secondary structures in mRNA are unlikely to become therapeutically applicable. Rather, a more viable approach is the use of low molecular size tetraplex unfolding agents. The cationic porphyrin 5,10,15,20-tetra(*N*-methyl-4-pyridyl)porphyrin (TMPyP4) whose size and geometry approximate those of a guanine quartet, was shown to selectively interact with and stabilize quadruplex structures formed by the guanine-rich sequences of telomeric DNA or within the NHE III₁ promoter of *c-MYC* (23,24). Conversely, however, this cationic porphyrin effectively destabilized *in vitro* G₂ bimolecular tetraplex structure of (CGG)_n in DNA and RNA (25). Owing to its quadruplex (CGG)_n disrupting activity, TMPyP4 might potentially serve as a model low molecular size agent that may increase the translation efficiency of premutation *FMRI* mRNA. Yet, being a charged molecule, TMPyP4 is unlikely to cross the blood–brain barrier and it thus cannot effectively treat neurodevelopmental disorders such as FXTAS. Use of this cationic porphyrin to destabilize d/r(CGG)_n quadruplexes *in vivo* constitutes, therefore, a proof of principle rather than identification of a clinically applicable therapeutic agent.

In this work we demonstrate that TMPyP4 unfolded tetraplex RNA (CGG)_n *in vitro* and enhanced the translation of 5' UTR (CGG)₉₉-*FL* mRNA in a reticulocyte lysate system. TMPyP4 that was added to HEK293 cells at excess over 5' UTR (CGG)₉₉-*FL* mRNA increased the efficiency of its translation. When introduced at a low ratio relative to the premutation mRNA, the cationic porphyrin failed to affect by itself the efficiency of translation. However, TMPyP4 together with sub-saturating amounts of either CBF-A or hnRNP A2 cooperatively augmented the efficiency of the *in vivo* translation of 5' UTR

(CGG)₉₉-*FL* mRNA. Potential therapeutic implications of these findings are discussed.

MATERIALS AND METHODS

Cationic porphyrins

The cationic porphyrin 5,10,15,20-tetra(*N*-methyl-4-pyridyl)porphyrin (TMPyP4) was the product of Calbiochem. Its two isomers 5,10,15,20-tetra(*N*-methyl-3-pyridyl)porphyrin (TMPyP3) and 5,10,15,20-tetra(*N*-methyl-2-pyridyl)porphyrin (TMPyP2) were the generous gift of Dr Lawrence H. Hurley (University of Arizona). Because of their photolability, stock solutions of 1.0 mM of each porphyrin in water were stored in opaque tubes at –70°C and diluted and used in the dark for *in vitro* or *in vivo* experiments. We established that the *in vivo* application of TMPyP4 at all its specified concentrations and under every described experimental condition did not affect cell viability or growth rate.

Plasmids

Plasmids: pT7-*FMRI*-5'-UTR(CGG)_n-*FL* (*n* = 30 or 99); pCS107; pCS107(CGG)₃₃; p*FMRI*-5'-UTR(CGG)_n-*FL* (*n* = 0, 30 or 99); pCMV2-Flag; pCMV2-Flag-*CBF-A* and pCMV2-Flag-*hnRNP A2* vectors that harbored genes that encode the quadruplex destabilizing proteins CBF-A and hnRNP A2, respectively, and a pCMV-*RL* plasmid (Promega) that encodes *Renilla reniformis* luciferase under the control of a CMV promoter were prepared and propagated as we recently detailed (19).

Electrophoretic resolution of 5'-³²P pCS107(CGG)₃₃ RNA

Total 1.0 μg of BstX1 linearized pCS107(CGG)₃₃ was transcribed *in vitro* at 37°C for 2 h in AmpliScribe SP6 system (Epicenter Biotechnologies) according to the manufacturer's instructions in a reaction mixture that contained 0.3 mCi [α-³²P]-UTP (3000 Ci/mmol). The reaction was terminated by the addition of SDS to a final concentration of 0.5%, and the product RNA was ethanol precipitated and washed as described (19). The air-dried RNA precipitate was suspended in 25 mM Tris–HCl buffer, pH 8.0 that contained 0.5 mM DTT, 1.0 mM EDTA and 20% glycerol. Following heating at 65°C for 3 min, cooling at 4°C for 2 min and incubation in the dark at room temperature for 10 min in the presence or absence of 20 mM KCl or LiCl and without or with 1.6 μM TMPyP4, RNA aliquots of equal radioactivity were resolved in the dark by electrophoresis through a non-denaturing 0.6% agarose gel. Positions of the migrating RNA samples relative to a ³²P-labelled RNA transcript of EcoR1 linearized pCS107 that was devoid of the (CGG)₃₃ tract, were identified by exposure of the dried gels to phosphor imager plates.

Translation *in vitro*

pT7-*FMRI*-5'-UTR(CGG)_n-*FL* plasmids (*n* = 30 or 99) were linearized by Xba cleavage and transcribed at 37°C for 90 min in an AmpliScribe™ T7-Flash™ Transcription kit (Epicentre Technologies) according to the

manufacturer's instructions. Following removal of the template DNA by digestion at 37°C for 15 min with RNase free DNase (Promega), the product RNA was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform extraction and ethanol precipitation. The centrifuged and air-dried RNA pellet was resuspended in RNase-free water and its amount was determined by electrophoretic resolution side-by-side with known amounts of tRNA. Aliquots of 0.5 µg RNA that included 30 or 99 5' (CGG) repeats were translated *in vitro* in a mixture that contained in a final volume of 25 µl: 12.5 µl rabbit reticulocyte lysate (Flexi translation system, Promega), 20 µM amino acids mixture, 2.0 mM DTT; 1.0 mM magnesium acetate; 70 mM KCl and 20 units of ribonuclease inhibitor (Takara) without or in the presence of increasing amounts of TMPyP4, TMPyP3 or TMPyP2. The mixtures were incubated at 30°C for 90–120 min and the translation reaction was terminated by rapid cooling of the samples to 4°C. FL activity was measured in 10 µl aliquots in a Glomax 20/20 luminometer (Promega) as we described (19).

Transfection of cultured human cells

Human Embryonic Kidney 293 (HEK293) cells were seeded in 0.1% gelatin-coated 10 cm plates and grown to 80–90% confluence at 37°C and in 5% CO₂ atmosphere in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 4.5 g/l D-glucose, 5.0 mM L-glutamine, 10% fetal calf serum, 83.3 U/ml each of penicillin and streptomycin and 0.2 mg/ml Amphotericin B (Biological Industries, Israel). The cells were detached by Trypsin-EDTA, reseeded in gelatin-coated 6-well plates at 3×10^5 to 5×10^5 cells/well and immediately co-transfected with three plasmids: p*FMRI*-5'-UTR(CGG)_n-*FL* ($n = 0, 30$ or 99) that harbored firefly luciferase (FL) reporter gene without or with 5'-(CGG)₃₀ or (CGG)₉₉ repeat tracts; pCMV-*RL* normalizing reporter vector and pCMV2-Flag-*CBF-A* or pCMV2-Flag-*hnRNP A2* plasmids that expressed the quadruplex destabilizing proteins CBF-A or hnRNP A2, respectively. Briefly, 6 µl of jetPEI DNA transfection reagent (Polyplus-Transfection) in 100 µl of 150 mM NaCl was added to equal volumes of mixtures that had low or high ratios of CBF-A encoding plasmid to the p*FMRI*-5'-UTR(CGG)_n-*FL* reporter plasmid. Mixtures with a low ratio of CBF-A encoding vector to reporter plasmid contained 500 ng p*FMRI*-5'-UTR(CGG)_n-*FL* ($n = 0, 30, 99$); 0.05 µg pCMV-*RL* and 2.5 µg pCMV2-Flag *CBF-A* in 150 mM NaCl. Similarly composed mixtures with a high ratio of CBF-A encoding vector to p*FMRI*-5'-UTR(CGG)_n-*FL* ($n = 0$ or 99) reporter plasmid contained 50 ng of p*FMRI*-5'-UTR(CGG)_n-*FL* ($n = 0$ or 99) and 2.9 µg pCMV-Flag *CBF-A* and 5 ng pCMV-*RL*. Mixtures with a low amount of hnRNP A2 encoding vector to the reporter plasmid contained 250 ng p*FMRI*-5'-UTR(CGG)_n-*FL* ($n = 0, 30, 99$) and 2.75 µg pCMV-Flag-*hnRNP A2* and 25 ng pCMV-*RL*. Following incubation at 37°C for 2 h, each plate was supplemented with 2.0 ml of growth medium and 1.0 mM TMPyP4 was added as specified to some plates to a final concentration of 20 µM.

The described procedure allowed for transfection efficiency of >90% as assessed by parallel monitoring GFP expression in cells that were similarly transfected with a GFP bearing vector. The cells were harvested 24 h post-transfection using Trypsin-EDTA and resuspended in 1.0 ml of cold growth medium. Aliquots of each sample were used to determine FL and RL activities and to conduct semi-quantitative reverse transcriptase (RT)-PCR measurements of the levels of their mRNA transcripts (see below).

Corrections for variations in cell viability and transfection efficiency were performed for each experiment as described (19) except that the RL-corrected FL activity was normalized to FL activity of cells transfected with reporter p*FMRI*-5'-UTR-*FL* vector with no upstream (CGG) repeat tract. Transfection efficiencies, as assessed by the measured levels of RL protein activity and RNA, were consistently unaffected by the size of the (CGG)_n tract in the co-transfected p*FMRI*-5'-UTR(CGG)_n-*FL* plasmid. Thus, for instance, in a typical series ($N = 5$) of RL measurements in cells that were co-transfected with an FL plasmids with no (CGG)_n tract or with a (CGG)₉₉ repeat, the respective average values of RL protein activity were $14\,045 \pm 5\,360$ and $16\,615 \pm 4\,010$ luminescence units and the measured levels of RL RNA were, respectively, 2082 ± 865 and 1832 ± 900 phosphor image pixels.

Dual luciferase assay

FL and RL activities were determined in lysates of transfected HEK293 cells according to the manufacturer's instructions using the dual luciferase reporter assay system (Promega). Briefly, the cells were lysed in passive lysis buffer (Promega) and 40 µl of cell lysate were added to 50 µl luciferase reagent II. Following a 2 s delay, FL activity was measured for 10 s in a Glomax 20/20 luminometer. The reaction was terminated by adding 50 µl Stop and Glo reagent to quench the FL activity and following a 2 s delay, the activity of RL was determined for 10 s.

Semi-quantitative RT-PCR measurement of relative mRNA levels

Total cell RNA was isolated from HEK-293 cells 24 h after transfection using Total RNA purification kit (Norgen Biotech Corporation). Contaminating plasmid and genomic DNA were removed from the isolated RNA by use of Turbo DNA-free kit (Ambion). For each sample, 0.5 µg of RNA was reverse-transcribed with Verso cDNA kit (Thermo Fisher Scientific) employing anchored Oligo-dT primers. To verify that all the amplification products were copies of the RNA template and not of contaminating DNA, every set of reactions included a negative control of a mixture without RT. After first-strand synthesis, the product cDNA was quantified by performing semi-quantitative PCR reaction. Each reaction mixture contained in a final volume of 25 µl: 0.025 µg cDNA; 0.3 mCi [α -³²P] dCTP (3000 Ci/mmol, Amersham) and 5.0 pmol each of the respective FL forward and reverse primers; 5'-d(GCTCCAACACC CCAACATCT)-3' and 5'-d(TTTCTTGCGTCGAGTT TTCC)-3' or the respective RL forward and reverse

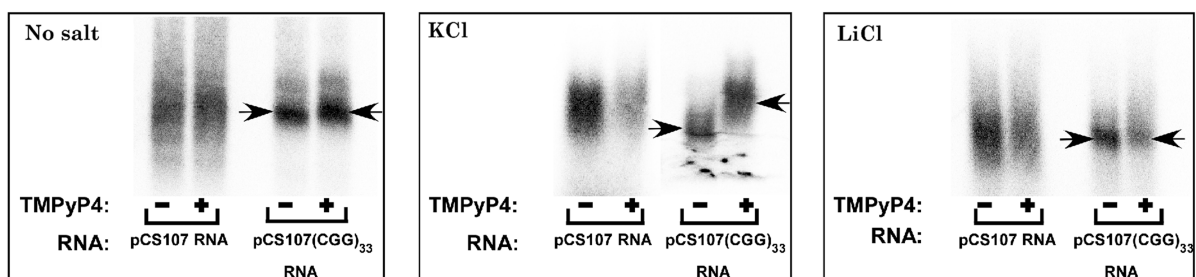


Figure 1. TMPyP4 destabilizes *in vitro* a secondary structure of 5'-(CGG)₃₃ containing RNA. Equal amounts of ³²P-labelled 365-nt-long pCS107(CGG)₃₃ RNA transcript were resolved by electrophoresis through a non-denaturing 0.6% agarose gel next to ³²P-labelled 339-nt-long marker pCS107 RNA transcript that lacked a (CGG)₃₃ tract. The RNA samples were pre-incubated at 4°C for 20 min in the absence or presence of 1.6 μM TMPyP4 and with or without 20 mM of KCl or LiCl followed by their electrophoresis in gels that, respectively, were devoid of salt or contained 20 mM of either KCl or LiCl. Shown are phosphor images of the dried gels.

primers; 5'-d(GGGATGAATGGCCTGATATTGAAGAAG)-3' and 5'-d(CAATTTGTACAACGTCAGGTTTACCACC)-3'. Measured levels of radiolabelled FL and RL reverse transcripts for each cycle between the 25th and 32th cycles showed the 27th or 30th to be the mid-points of the respective linear amplification range of FL and RL RNA. Twice repeated and averaged standard PCR procedures were conducted with FL or RL cDNA for 27 or 30 cycles, respectively, each of 20 s at 94°C followed by 25 s at 58°C and 1 min at 72°C. To verify that the amplification products were authentic FL or RL DNA and not copies of contaminating DNA, every set of reactions included a negative control reaction mixture that lacked first-strand cDNA.

Western analysis

HEK293 cells that were co-transfected with pCMV2-Flag-CBF-A or pCMV2-Flag-hnRNP A2 and pFMR1-5'-UTR(CGG)_n-FL (*n* = 0, 30 or 99) without or with exposure to TMPyP4 as specified, were harvested 24 h after the transfection and lysed. Equal amounts of the cell lysate protein were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane and the expression of Flag-CBF-A or Flag hnRNP A2 was detected by a murine anti-Flag primary antibody (Sigma) with horseradish peroxidase-conjugated goat anti mouse IgG (H + L, Pierce) serving as the secondary antibody (19). Horseradish peroxidase activity was monitored using a Super We Pico chemiluminescence substrate (Pierce).

RESULTS

TMPyP4 untangles *in vitro* an intramolecular quadruplex structure of r(CGG)₃₃

We reported previously that a (CGG)₃₃ repeat sequence within an RNA transcript of pCS107(CGG)₃₃ formed in a K⁺ ion-dependent reaction a compact, T1 ribonuclease resistant secondary structure whose properties were consistent with those of an intramolecular quadruplex (19). In a parallel line of investigation we found that the cationic porphyrin TMPyP4 destabilized *in vitro* bimolecular tetraplex (CGG)_n structures in DNA and RNA (25). Here we inquired whether TMPyP4 was also capable of

unfolding an intramolecular r(CGG)_n quadruplex. To this end, ³²P-labelled RNA transcript of pCS107(CGG)₃₃ was incubated without or with 5.0 μM TMPyP4 in the absence of alkali ion or in the presence of 20 mM of either KCl or LiCl which, respectively, promote or do not support the formation of stable quadruplex structures of DNA or RNA. To assess the effect of TMPyP4 on potential secondary structure of the pCS107(CGG)₃₃ RNA, its electrophoretic mobility in a non-denaturing agarose gel was compared to the migration of marker pCS107 RNA that lacked the repeat sequence. As seen in Figure 1, the mobility of the marker RNA molecules remained unaffected by TMPyP4 in the absence of alkali ion or in the presence of either KCl or LiCl. Similarly, TMPyP4 did not affect the electrophoretic migration of pCS107(CGG)₃₃ RNA both in the absence of salt or in a buffer that contained Li⁺ ions (Figure 1, left and right panels, respectively). In contrast, the relative electrophoretic mobility of the (CGG)₃₃ containing RNA was increased in the presence of K⁺ ions and TMPyP4 slowed its migration (Figure 1, middle panel). At the same time, the mobility of the pCS107 RNA that did not contain a (CGG) repeat tract remained unchanged in the presence of K⁺ ions without or with TMPyP4. Thus, the likeliest cause of the slowed relative electrophoretic mobility of the pCS107(CGG)₃₃ RNA was the unfolding by TMPyP4 of a rapidly migrating compact intramolecular secondary structure of the r(CGG)₃₃ tract. Potassium ions are essential for the formation and stability of quadruplex nucleic acids whereas lithium is unable to promote its generation and stability (26,27). Hence, the ability of TMPyP4 to slow the mobility of pCS107(CGG)₃₃ in K⁺-containing buffer but not in the presence of Li⁺ ions conforms with a suggestion that the compact structure of pCS107(CGG)₃₃ was a quadruplex. Yet, the possibility that the compact RNA formation represented a different secondary structure such as hairpin cannot be excluded (see 'Discussion' section).

TMPyP4 enhances the translation *in vitro* of (CGG)₉₉-FL mRNA

Evidence indicated that translation was impeded by quadruplex structures of a FXS premutation (CGG)_n stretch in mRNA (19) and by tetraplex formations of G-rich tracts in various other mRNA molecules (20–22). We thus

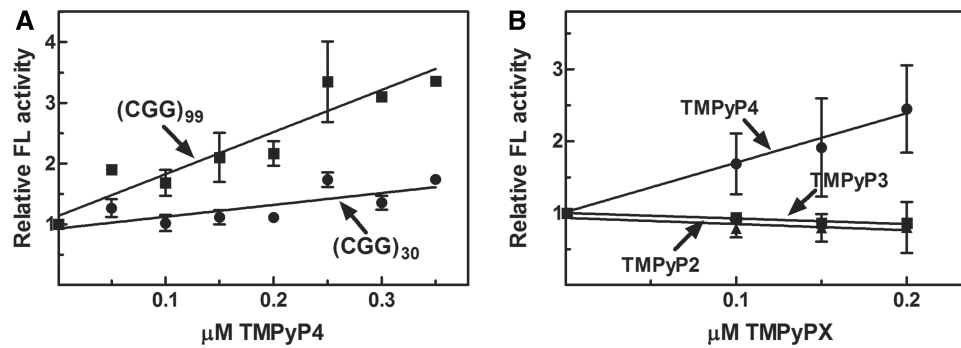


Figure 2. TMPyP4, but not TMPyP2 or TMPyP3, enhances the *in vitro* translation of (CGG)₉₉-FL mRNA. (A) Equal amounts of ³²P-labelled mRNA transcripts of pT7-*FMRI*-5'-UTR(CGG)_n-FL ($n = 30$ or 99) were translated *in vitro* in a reticulocyte lysate system that contained increasing amounts of TMPyP4 and the activity of FL was determined as described under 'Materials and Methods' section. Presented are end-points of each reaction. (B) FL activity produced by the *in vitro* translation of (CGG)₉₉-FL mRNA in the presence of the indicated increasing amounts of the positional isomers TMPyP2, TMPyP3 or TMPyP4. Shown are end-points of each reaction.

inquired whether by virtue of its capability to unfold quadruplex r(CGG)_n, TMPyP4 was also able to remove the obstruction to translation. Equal amounts of mRNA transcripts of pT7-*FMRI*-5'-UTR(CGG)₃₀-FL or pT7-*FMRI*-5'-UTR(CGG)₉₉-FL that, respectively, included normal or premutation numbers of (CGG) repeats were translated in a reticulocyte lysate system that contained rising concentrations of TMPyP4. Determination of the relative activity of FL indicated that translation of pT7-*FMRI*-5'-UTR(CGG)₃₀-FL mRNA was only minimally enhanced in the presence of increasing amounts of TMPyP4. In contrast, the translation of pT7-*FMRI*-5'-UTR(CGG)₉₉-FL mRNA was augmented up to >3-fold in direct proportion to the concentration of added TMPyP4 (Figure 2A). These results were in line with the reported minimal effect of the quadruplex unfolding proteins hnRNP A2 and CBF-A on the *in vivo* translation of (CGG)₃₀-FL mRNA and their robust stimulation of the translation of premutation (CGG)₉₉-FL mRNA (19). Results presented in Figure 2B showed that in contrast to the stimulation of the translation of (CGG)₉₉-FL mRNA by TMPyP4, protein synthesis was slightly depressed by its two positional isomers TMPyP2 and TMPyP3 that were shown to be incapable of destabilizing quadruplex r(CGG)_n (25). Conceivably, therefore, the enhancement of FL synthesis by TMPyP4 was a consequence of its ability to destabilize a translation blocking r(CGG)₉₉ quadruplex structure at the 5' UTR terminus of the FL mRNA.

The quadruplex (CGG)_n destabilizing agents TMPyP4 and CBF-A increase the efficiency of the *in vivo* translation of (CGG)₉₉-FL mRNA

We next inquired whether TMPyP4 alone or in combination with tetraplex r(CGG)_n unfolding hnRNPs might increase the efficiency of the *in vivo* translation of premutation (CGG)_n mRNA. To this end we first defined experimental conditions under which separately introduced agents, TMPyP4 or CBF-A, affected the efficacy of translation of (CGG)₉₉-FL mRNA in living cells. Since the amount of (CGG)₉₉-FL mRNA was proportional to the amount of transfecting reporter plasmid (data not shown),

we empirically determined conditions for the introduction of TMPyP4 or CBF-A at low or high ratios to the reporter plasmids and consequently to their mRNA transcripts. Matched cultures of HEK293 cells were co-transfected with either p*FMRI*-5'-UTR(CGG)₀-FL or p*FMRI*-5'-UTR(CGG)₉₉-FL reporter plasmids and with a pCMV-*RL* normalizing vector. The transfected cells were either exposed to TMPyP4 or were co-transfected with the CBF-A expressing vector pCMV2-Flag *CBF-A*. Control cells were transfected with reporter and normalizing plasmids but were not exposed to either TMPyP4 or CBF-A. Following a 24 h period of growth, the cells were lysed and RL-normalized levels of FL activity and FL mRNA were measured in the cell lysates. Table 1 summarizes the results of multiple independent determinations of the levels of FL protein and mRNA obtained in cells that expressed (CGG)₉₉-FL mRNA relative to similarly treated cells that expressed (CGG)₀-FL mRNA. As is evident, the respective efficiency of the *in vivo* translation of (CGG)₉₉-FL mRNA was increased by 2.8- or 3.2-fold when TMPyP4 or CBF-A were introduced at apparent excess over the reporter plasmid. Yet, no significant enhancement was observed when either agent was introduced at what were conceivably their sub-saturating amounts relative to the level of the (CGG)₉₉-FL mRNA (Table 1).

Notably, the elevated amount of premutation mRNA was decreased upon exposure of the cells to high relative doses of TMPyP4 or CBF-A (Table 1). This effect raised the possibility that in addition to being able to increase the efficiency of premutation mRNA translation, these agents might alleviate the presumed toxicity of premutation *FMRI* mRNA in FXS carrier cells by depressing its excessive accumulation (see 'Discussion' section).

High relative amounts of TMPyP4 together with CBF-A additively increase the efficiency of the *in vivo* translation of (CGG)₉₉-FL mRNA

A previous report (19) and results presented in Table 1 indicated that the efficacy of the *in vivo* translation of (CGG)₉₉-FL mRNA was increased when cells expressed high relative amounts of CBF-A or when they were

Table 1. Effect of different relative levels of TMPyP4 and CBF-A on the efficiency of the *in vivo* translation of (CGG)₉₉-FL mRNA

Agent	Low ratio of agent to pFMRI-5'-UTR(CGG) ₉₉ -FL ^a				High ratio of agent to pFMRI-5'-UTR(CGG) ₉₉ -FL ^b			
	FL protein	FL mRNA	FL protein/ mRNA [M] ^c	X-fold increase of translation efficacy	FL protein	FL mRNA	FL protein/ mRNA [N] ^c	X-fold increase of translation efficacy
–	4.9 ± 1.0	6.3 ± 1.9	0.8 [9]	1.0	4.6 ± 0.1	8.2 ± 2.7	0.6 [5]	1.0
TMPyP4	4.8 ± 1.0	5.8 ± 1.0	0.8 [8]	1.0	5.2 ± 0.7	3.1 ± 0.4	1.7 [4]	2.8
CBF-A	8.8 ± 1.2	8.6 ± 1.6	1.0 [5]	1.25	9.3 ± 1.9	5.0 ± 2.1	1.9 [4]	3.2

Listed are average levels and standard deviations of the of FL protein and mRNA relative to values obtained for HEK293 cells that were transfected with pFMRI-5'-UTR(CGG)₀-FL reporter plasmid and that were similarly exposed to TMPyP4, co-transfected with a CBF-A expressing plasmid or left untreated.

^a5 × 10⁵ HEK293 cells were transfected with 500 ng DNA of a pFMRI-5'-UTR(CGG)₉₉-FL reporter plasmid were either exposed to 20 μM TMPyP4 or co-transfected with 2.5 μg DNA of a CBF-A expressing pCMV2-Flag-CBF-A vector.

^b5 × 10⁵ HEK293 cells were transfected with 50 ng DNA of a pFMRI-5'-UTR(CGG)₉₉-FL reporter plasmid were either exposed to 20 μM TMPyP4 or co-transfected with 2.9 μg DNA of a CBF-A expressing pCMV2-Flag-CBF-A vector.

^cN: number of independent experiments.

AGENT	FL protein	FL mRNA	FL protein / mRNA [N]	X-fold enhancement
---	4.6 ± 0.1	8.2 ± 2.7	0.6 [4]	---
TMPyP4	5.2 ± 0.7	3.1 ± 0.4	1.7 [5]	2.8
CBF-A	9.3 ± 1.6	5.0 ± 2.1	1.9 [4]	3.2
TMPyP4 + CBF-A	14.0 ± 1.6	4.5 ± 1.7	3.1 [4]	5.2

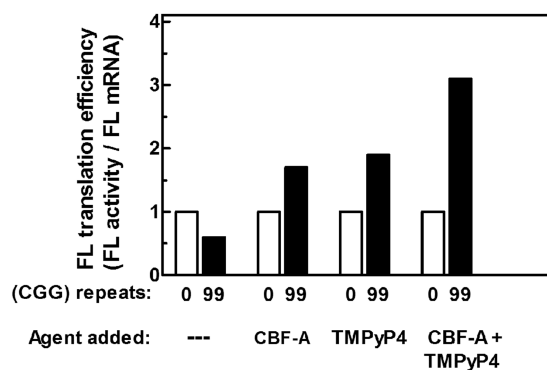


Figure 3. High relative amounts of TMPyP4 and CBF-A cooperate to additively increase the *in vivo* efficiency of translation of (CGG)₉₉-FL mRNA. HEK293 cells were transfected with 50 ng of either pFMRI-5'-UTR(CGG)₀-FL or pFMRI-5'-UTR(CGG)₉₉-FL reporter plasmids and with a pCMV-RL normalizing vector. Matched cultures were exposed to 20 μM TMPyP4, co-transfected with 2.9 μg pCMV2-Flag CBF-A plasmid or were both treated with TMPyP4 and co-transfected with pCMV2-Flag-CBF-A. Following cell growth, levels of FL activity and mRNA were determined as described under 'Materials and Methods' section and in Table 1. Tabulated are average values ± SD of the indicated number (N) of independent determinations of the translation efficiency, (FL activity divided by FL mRNA), of (CGG)₉₉-FL mRNA relative to (CGG)₀-FL mRNA. Bottom: Graphic presentation of the results.

exposed to a relative excess of TMPyP4. Based on these results we examined the effect of combining high relative levels of TMPyP4 and the quadruplex unfolding CBF-A protein on the efficacy of the *in vivo* translation of (CGG)₉₉-FL mRNA. HEK293 cells were transfected with 50 ng of either pFMRI-5'-UTR(CGG)₀-FL or pFMRI-5'-UTR(CGG)₉₉-FL reporter plasmids and with a pCMV-RL normalizing vector. Parallel cultures were exposed to 20 μM TMPyP4, co-transfected with 2.9 μg pCMV2-Flag CBF-A plasmid or were both treated with TMPyP4 and co-transfected with pCMV2-Flag-CBF-A. The selected high relative ratios of TMPyP4 or CBF-A encoding vector to the reporter FL plasmid were those indicated in the respective section of Table 1. Cells were

grown for 24h, lysed and RL normalized levels of FL activity and FL mRNA were determined in lysates of the differently treated cells and in control cells that were transfected by the respective reporter plasmids but were not exposed to any quadruplex unfolding agent. The relative translation efficacies in the absence or presence of quadruplex destabilizing agents were assessed by measuring the ratio of expressed FL protein activity to FL mRNA. Results shown in Figure 3 show the translation efficiencies of (CGG)₉₉-FL mRNA relative to efficiencies of translation of (CGG)₀-FL mRNA in equally treated cells.

The obtained data indicated that the relative efficiency of translation of (CGG)₉₉-FL mRNA in cells that were

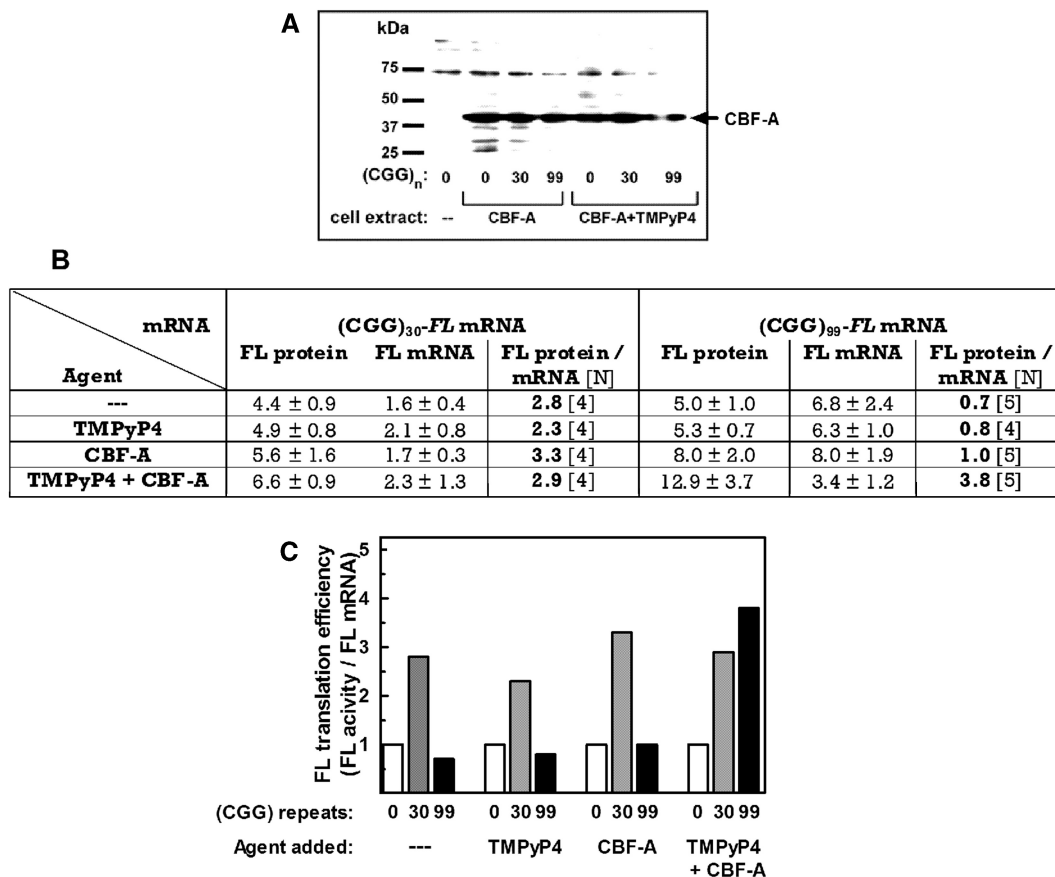


Figure 4. Low relative amounts TMPyP4 and CBF-A cooperate to synergistically increase the *in vivo* efficiency of translation of (CGG)₉₉-FL mRNA. (A) Western blot analysis of CBF-A expression in HEK293 cells that were co-transfected with 2.5 μ g DNA of CBF-A expressing plasmid and 500 ng DNA of reporter FL plasmid that contained 30, 99 or no (CGG) repeats and that were or were not exposed to 20 μ M TMPyP4. (B) Relative translation efficiencies of (CGG)₀-FL mRNA, (CGG)₃₀-FL mRNA or (CGG)₉₉-FL mRNA without or in the presence of sub-saturating amount of TMPyP4 or CBF-A alone or a combination thereof. Displayed are average results \pm SD of the indicated number (N) of independent experiments. (C) Graphic presentation of results tabulated in (B).

exposed to TMPyP4 or expressed CBF-A was, respectively, increased by 2.8- or 3.2-fold. Introduction of a combination of the two quadruplex destabilizing agents together increased the relative efficacy of translation by 5.2-fold (Figure 3). Thus, the magnitude of the stimulation that was attained in the presence of both TMPyP4 and CBF-A was nearly a sum of the enhancement by each agent separately. The additive effect of combining TMPyP4 with CBF-A suggested that the two agents shared a common mechanism of translation augmentation (see 'Discussion' section).

Combination of low relative amounts of TMPyP4 and CBF-A synergistically increases the *in vivo* efficiency of translation of (CGG)₉₉-FL mRNA

Although sub-saturating levels of TMPyP4 or CBF-A each failed to raise the efficacy of the *in vivo* translation of pre-mutation (CGG)₉₉-FL mRNA (Table 1), we speculated that combining the two agents might increase the translation efficiency of pre-mutation (CGG)₉₉ mRNA. To examine this possibility, matched HEK293 cell cultures were co-transfected with 500 ng of

pFMRI-5'-UTR(CGG)_n-FL ($n = 0, 30$ or 99) reporter plasmids and a *pCMV-RL* normalizing vector. Parallel to transfected control cells that were left untreated, matching cultures were exposed to 20 μ M TMPyP4, co-transfected with 2.5 μ g *pCMV2-Flag CBF-A* or treated by a combination thereof. The chosen low relative ratios of TMPyP4 or CBF-A expressing vector to the reporter FL plasmid were those indicated in the respective section of Table 1. Following a 24 h growth period, the cells were harvested and lysed and RL-normalized levels of expressed FL protein and mRNA were determined. Relative translation efficacies were calculated by dividing the measured efficiencies of translation of (CGG)₃₀ or (CGG)₉₉ containing mRNA by the values obtained for (CGG)₀ mRNA in similarly treated cells.

Western analysis indicated that CBF-A expression was maintained at an unchanged level regardless of the number of (CGG) repeats in the FL reporter vector or the absence or presence of TMPyP4 (Figure 4A). However, combining TMPyP4 with CBF-A affected differently the relative translation efficacies of FL mRNA molecules that contained 30 or 99 (CGG) repeats. In line with our previous report (19), (CGG)₃₀-FL mRNA

was translated at a ~3-fold higher efficiency than *FL* mRNA with no repeat tract (Figure 4B and C). This level of (CGG)₃₀-*FL* mRNA utilization was not affected by either the expression of sub-saturating amounts of CBF-A or the addition of TMPyP4. A slight enhancement of the translation efficiency observed upon introduction of both agents together was not statistically significant (Figure 4B and C). As reported (19), the presence of a premutation 5'-UTR (CGG)₉₉ repeat sequence in *FL* mRNA depressed its utilization relative to *FL* mRNA molecules with no repeat tract and particularly relative to (CGG)₃₀ containing mRNA (Figure 4B and C). Furthermore, this diminished efficiency of translation was not elevated by sub-saturating amounts of either TMPyP4 or CBF-A. Most conspicuously, however, cells that expressed sub-saturating amounts of CBF-A and were also exposed to low relative concentration of TMPyP4 utilized (CGG)₉₉-*FL* mRNA at >5-fold greater efficiency than cells that were not exposed to any quadruplex unfolding agent (Figure 4B and C). Hence, applying to cells a combination of sub-saturating amounts of TMPyP4 and CBF-A that are not effective by themselves, resulted in synergistic enhancement of the efficiency of translation of (CGG)₉₉-*FL* mRNA.

The efficiency of the *in vivo* translation of (CGG)₉₉-*FL* mRNA is also increased synergistically by combining low relative amounts of hnRNP A2 and TMPyP4

Similarly to CBF-A, hnRNP A2 was shown to be capable of unfolding *in vitro* quadruplex structures of (CGG)_{*n*} in DNA and RNA (18) and to increase the efficacy of the *in vivo* translation of premutation (CGG)₉₉ mRNA (19). We next inquired whether the synergistic enhancement of translation by TMPyP4 in combination with CBF-A can be reproduced by replacing CBF-A with hnRNP A2. In preparatory experiments similar to those summarized in Table 1, we defined high and low ratios of hnRNP A2 expressing vector to *FL* reporter plasmid that, respectively, enhanced the translation of (CGG)₉₉-*FL* mRNA or elicited no effect (data not shown). In the following experiment we expressed hnRNP A2 at ineffective low relative ratio relative to the reporter plasmids. Matched HEK293 cell cultures were co-transfected with 250 ng of p*FMRI*-5'-UTR(CGG)_{*n*}-*FL* (*n* = 0, 30 or 99) reporter plasmids and a pCMV-*RL* normalizing vector. Transfected control cells were left untreated whereas parallel cultures were exposed to 20 μM TMPyP4, co-transfected with 2.75 μg DNA of the hnRNP A2 expressing vector pCMV2-Flag *hnRNP A2* or treated by a combination of both agents. Cells were grown and lysed and *RL*-normalized relative levels of *FL* activity and mRNA were measured as described in the preceding section. Western analysis indicated that the expression of hnRNP A2 was unaffected by the number of (CGG) repeats in the reporter plasmid or by the absence or presence of TMPyP4 (Figure 5A). Results indicated that the efficiency of translation of (CGG)₃₀-*FL* mRNA was elevated by 2-fold relative to *FL* mRNA with no repeat tract and that exposure to sub-saturating doses of TMPyP4 or hnRNP A2 or to a combination thereof did not

significantly alter its efficiency of translation (Figure 5B and C). In contrast, as documented (Table 1, Figures 3 and 4), the presence of 99 5'-(CGG) repeats in *FL* mRNA depressed the efficiency of its translation. Also, this diminished efficacy was not elevated by separately introduced sub-saturating amounts of TMPyP4 or hnRNP A2. However, the translation efficiency of (CGG)₉₉-*FL* mRNA was higher by 1.4–1.9-fold in cells that were exposed to a combination of sub-saturating amounts of TMPyP4 together with hnRNP A2 (Figure 5B and C). Thus, similarly to CBF-A, sub-saturating levels of hnRNP A2 cooperated with low relative amounts of TMPyP4 to synergistically increase the efficiency of the *in vivo* translation of mRNA molecules that included a 5' (CGG)₉₉ premutation tract.

DISCUSSION

Expansion of the *FMRI* 5'-UTR repeat tract to >55–200 copies of the (CGG) trinucleotide causes, in subgroups of female and male carriers of FXS premutation, respective development of POF (4,6) and of FXTAS (7–9). Although the premutation state is characterized by elevated synthesis and accumulation of 5–10-fold higher than normal amounts of *FMRI* mRNA molecules (10–12), levels of their product protein FMRP are reduced or remain unchanged (11,14). We have shown recently that impediment to translation by quadruplex structures of the 5'-UTR (CGG)_{>55–200} premutation tract in *FMRI* mRNA is the likely source of the apparent diminished efficacy of FMRP synthesis (19). Also, over-expression in living cells of two d/r(CGG)_{*n*} quadruplex destabilizing hnRNPs; CBF-A and hnRNP A2, (18,19,28,29) removed the obstruction to translation and reinstated normal efficiency of premutation mRNA utilization (19). This finding suggested that quadruplex (CGG)_{*n*} unfolding agents may be useful in partly overcoming the molecular defect in cells of premutation carriers. However, therapeutic utilization of exogenously introduced proteins such as CBF-A or hnRNP A2 is impractical and a more realistic option is the use of small quadruplex destabilizing molecules. We thus chose to examine the cationic porphyrin TMPyP4 that, contrary to its capability to stabilize quadruplex structures of the telomeric repeat (23,25,30) and of a guanine-rich tract in the *c-Myc* promoter (24), was shown to paradoxically destabilize d(CGG)_{*n*} quadruplexes (25).

In the present work we first demonstrated that TMPyP4 unfolded *in vitro* an intramolecular secondary structure of an r(CGG)₃₃ tract (Figure 1). Since RNA that did not contain r(CGG)₃₃ repeat tract was unaffected by TMPyP4 and as the untangled pCS107(CGG)₃₃ RNA secondary structure was generated in the presence of K⁺ but not Li⁺ ions, we propose that it represented a quadruplex. In such case, similarly to its reported ability to resolve *in vitro* a formally defined bimolecular tetraplex structure of r(CGG)_{*n*} (25), TMPyP4 appeared to be able to unfold *in vitro* an intramolecular quadruplex form of the RNA repeat sequence. It should be cautioned, however, that although previous additional indirect evidence also indicated that (CGG)_{*n*} RNA formed an intramolecular

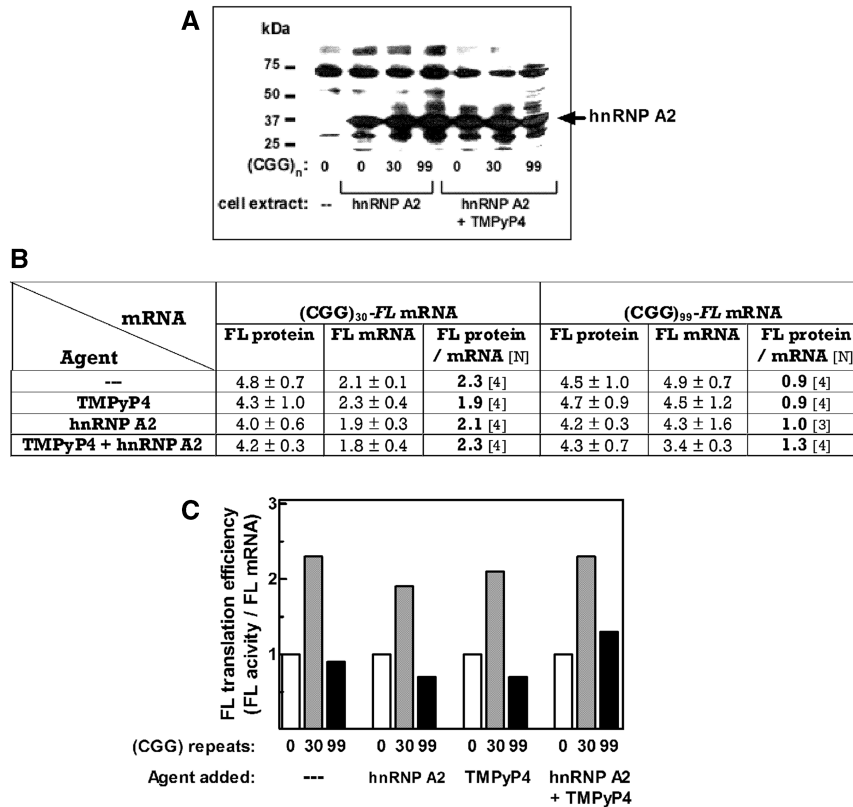


Figure 5. Low relative amounts TMPyP4 and hnRNP A2 cooperate to synergistically increase the *in vivo* efficiency of translation of (CGG)₉₉-FL mRNA. (A) Western blot analysis of hnRNP A2 expression in HEK293 cells that were co-transfected with 2.75 μ g DNA of hnRNP A2 expressing plasmid and 250 ng DNA of reporter FL plasmid that contained 30, 99 or no (CGG) repeats and that were or were not exposed to 20 μ M TMPyP4. (B) Relative translation efficiencies of 5'UTR (CGG)₀-FL mRNA 5'UTR (CGG)₃₀-FL mRNA or 5'UTR (CGG)₉₉-FL mRNA without or in the presence of sub-saturating amount of TMPyP4 or hnRNP A2 alone or a combination thereof. Displayed are average results \pm SD of the indicated number (N) of independent experiments. (C) Graphic presentation of results tabulated in (B).

quadruplex (19), a formal proof of the nature of the r(CGG)₃₃ secondary structure is still lacking.

TMPPyP4, but not its positional isomers TMPyP2 or TMPyP3 that are unable to unfold quadruplex (CGG)_n (25), enhanced the translation in reticulocyte lysate of a premutation-like reporter (CGG)₉₉-FL mRNA (Figure 2). Based on these findings we compared the ability of TMPyP4 or of the quadruplex unfolding proteins CBF-A and hnRNP A2 to increase the efficiency of the *in vivo* translation of premutation (CGG)₉₉-FL mRNA. Our initial results indicated that exposure of HEK293 cells to TMPyP4, at empirically determined excess over the pFMR1-5'-UTR(CGG)₉₉-FL transfecting reporter plasmid, elevated the efficiency of (CGG)₉₉-FL mRNA translation (Table 1). The extent of TMPyP4-induced stimulation was comparable to the enhancement of *in vivo* translation by CBF-A [Table 1; (20)]. In contrast, lower amounts of TMPyP4, CBF-A or hnRNP A2 relative to the reporter pFMR1-5'-UTR(CGG)₉₉-FL plasmid failed to increase the efficiency of (CGG)₉₉-FL mRNA translation (Table 1 and Figure 5). Since the amount of (CGG)₉₉-FL mRNA was proportional to the amount of transfecting reporter plasmid, it appeared that in order to enhance translation both TMPyP4 and CBF-A had to be present at an excess over the premutation mRNA.

The required TMPyP4 to RNA stoichiometry and the non-catalytic quadruplex nucleic acid destabilizing activity of CBF-A (18,19,28,29) suggested that the two agents had a similar mechanism of action. Data summarized in Figure 3 that demonstrated additive augmentation of translation efficacy by TMPyP4 and CBF-A was indeed supportive of the supposition that these agents shared a similar mechanism of destabilization of quadruplex (CGG)_n structure in premutation mRNA. Conceivably, both the cationic porphyrin and CBF-A recognized G-quartets that were formed within the (CGG)_n sequence, bound to the quadruplex domain and diminished its stability, as reflected by the capacity of TMPyP4 to lower the melting temperatures of tetraplex (CGG)_n (25).

Our results indicated that the efficacy of translation of (CGG)₃₀-FL mRNA was up to 3-fold higher than that of (CGG)₀-FL mRNA (Figures 4B and 5B). A 5'-(CGG)₃₀ tract represents the most common number of (CGG) repeats in the FMR1 gene in the normal human population (31). It may be speculated that this size of the repeat sequence was evolutionarily conserved because it affords higher efficacy of FMRP synthesis, possibly by attracting translation factors to the FMR1 mRNA. Notably, however, the quadruplex disrupting agents TMPyP4, CBF-A or hnRNP A2 each by themselves or TMPyP4 together

with either protein did not alter the *in vivo* efficacy of translation of (CGG)₃₀-FL mRNA (Figures 4B and 5B). We assume, therefore, that unlike a quadruplex structure of a premutation-size 5'-(CGG)₉₉ tract, the shorter 5'-(CGG)₃₀ sequence either did not block translation or it even promoted it. Alternatively, the 5'-(CGG)₃₀ stretch may have been unstable at 37°C within the cells such that it was maintained as a single-strand also in the absence of quadruplex destabilizing agents.

Introduction of excessive amounts of TMPyP4 or of greatly over-expressed quadruplex disrupting hnRNPs is likely to be therapeutically impractical. Presented evidence showed, however, that although low relative amounts of these agents failed to stimulate by themselves the *in vivo* translation of (CGG)₉₉-FL mRNA, combining TMPyP4 with CBF-A (Figure 4) or with hnRNP A2 (Figure 5) elicited significant increases in the efficacy of translation of the premutation mRNA. The inability of a low relative dose of TMPyP4 to enhance by itself the translation of (CGG)₉₉-FL mRNA suggested that the levels of available endogenous CBF-A and hnRNP A2 in HEK293 cells were insufficient to cooperate with the externally added TMPyP4. It is possible however, that TMPyP4 alone may suffice to enhance the translation of premutation mRNA in other cell types that endogenously produce higher amounts of hnRNPs.

It has been suggested that the source of neurodegeneration in FXTAS is the accumulation of excessive amounts of premutation *FMR1* mRNA in FXS carrier cells. A proposed RNA gain-of-function toxicity model suggested that the accumulated *FMR1* mRNA molecules bind r(CGG) repeat binding proteins (RBPs) that are thus sequestered from their normal functions (32,33). Interestingly, hnRNP A2/B1 was recently identified as a major RBP in a transgenic *Drosophila* model of FXTAS (34,35), providing independent evidence for the *in vivo* interaction of this quadruplex r(CGG)_n disrupting protein with the premutation repeat tract. In addition to its association with premutation (CGG)_n mRNA and its capacity to enhance its translation, over expressed hnRNP A2 was shown to diminish the amount of the accumulated mRNA (19). Data presented in this manuscript indicated that the elevated amount of premutation mRNA was also reduced in cells that were exposed to TMPyP4 or to CBF-A (Table 1 and Figure 4). It should be noted that both agents had to be introduced in excess over the (CGG)₉₉-FL mRNA. Introduction of TMPyP4, CBF-A or hnRNP A2 alone, each at low ratio relative to the premutation mRNA, did not decrease the level of (CGG)₉₉-FL mRNA (Table 1, Figures 4 and 5). Only combinations of low ratios of the porphyrin with either CBF-A or hnRNP A2 depressed mRNA accumulation. Low ratios of the quadruplex disrupting porphyrin or hnRNPs by themselves to premutation mRNA also failed to elicit enhancement of translation whereas combinations of TMPyP4 with either protein both augmented the translation of 5'-(CGG)₉₉-mRNA and depressed its accumulation (Table 1, Figures 4 and 5). It is tempting, therefore, to implicate the quadruplex destabilizing activity of the proteins and TMPyP4 in both translation enhancement and lowering of the level of premutation mRNA. In any

case, whatever the mechanism is by which TMPyP4 and the two hnRNPs depressed mRNA accumulation, our observations suggested that in addition to being able to increase the efficiency of premutation mRNA translation, these agents may also be effective in decreasing the RNA toxicity in cells of FXS carriers.

ACKNOWLEDGEMENTS

We thank the two anonymous reviewers for their constructive critique.

FUNDING

Israel Science Foundation, United States-Israel Binational Science Foundation; the Fund for Promotion of Research at the Technion (to M.F.). Funding for open access charge: Israel Science Foundation research grant.

Conflict of interest statement. None declared.

REFERENCES

1. Penagarikano, O., Mulle, J.G. and Warren, S.T. (2007) The pathophysiology of fragile X syndrome. *Annu. Rev. Genomics Hum. Genet.*, **8**, 109–129.
2. Garber, K.B., Visootsak, J. and Warren, S.T. (2008) Fragile X syndrome. *Eur. J. Hum. Genet.*, **16**, 666–672.
3. Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T. and Nelson, D.L. (1991) Absence of expression of the *FMR-1* gene in fragile X syndrome. *Cell*, **66**, 817–822.
4. Coffey, S.M., Cook, K., Tartaglia, N., Tassone, F., Nguyen, D.V., Pan, R., Bronsky, H.E., Yuh, J., Borodyanskaya, M., Grigsby, J. et al. (2008) Expanded clinical phenotype of women with the *FMR1* premutation. *Am. J. Med. Genet. A*, **146A**, 1009–1016.
5. Hagerman, P.J. and Hagerman, R.J. (2004) The fragile-X premutation: a maturing perspective. *Am. J. Hum. Genet.*, **74**, 805–816.
6. Allingham-Hawkins, D.J., Babul-Hirji, R., Chitayat, D., Holden, J.J., Yang, K.T., Lee, C., Hudson, R., Gorwill, H., Nolin, S.L., Glicksman, A. et al. (1999) Fragile X premutation is a significant risk factor for premature ovarian failure: the International Collaborative POF in Fragile X study—preliminary data. *Am. J. Med. Genet.*, **83**, 322–325.
7. Hagerman, R.J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., Grigsby, J., Gage, B. and Hagerman, P.J. (2001) Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile X. *Neurology*, **57**, 127–130.
8. Berry-Kravis, E., Lewin, F., Wu, J., Leehey, M., Hagerman, R., Hagerman, P. and Goetz, C.G. (2003) Tremor and ataxia in fragile X premutation carriers: blinded videotape study. *Ann. Neurol.*, **53**, 616–623.
9. Amiri, K., Hagerman, R.J. and Hagerman, P.J. (2008) Fragile X-associated tremor/ataxia syndrome: an aging face of the fragile X gene. *Arch. Neurol.*, **65**, 19–25.
10. Tassone, F., Hagerman, R.J., Taylor, A.K., Gane, L.W., Godfrey, T.E. and Hagerman, P.J. (2000) Elevated levels of *FMR1* mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am. J. Hum. Genet.*, **66**, 6–15.
11. Kenneson, A., Zhang, F., Hagedorn, C.H. and Warren, S.T. (2001) Reduced FMRP and increased *FMR1* transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. *Hum. Mol. Genet.*, **10**, 1449–1454.
12. Tassone, F., Beilina, A., Carosi, C., Albertosi, S., Bagni, C., Li, L., Glover, K., Bentley, D. and Hagerman, P.J. (2007) Elevated *FMR1* mRNA in premutation carriers is due to increased transcription. *RNA*, **13**, 555–562.
13. Brouwer, J.R., Mientjes, E.J., Bakker, C.E., Nieuwenhuizen, I.M., Severijnen, L.A., Van der Linde, H.C., Nelson, D.L., Oostra, B.A. and

- Willemsen, R. (2007) Elevated Fmr1 mRNA levels and reduced protein expression in a mouse model with an unmethylated Fragile X full mutation. *Exp. Cell Res.*, **313**, 244–253.
14. Primerano, B., Tassone, F., Hagerman, R.J., Hagerman, P., Amaldi, F. and Bagni, C. (2002) Reduced FMR1 mRNA translation efficiency in fragile X patients with premutations. *RNA*, **8**, 1482–1488.
 15. Feng, Y., Zhang, F., Lokey, L.K., Chastain, J.L., Lakkis, L., Eberhart, D. and Warren, S.T. (1995) Translational suppression by trinucleotide repeat expansion at *FMR1*. *Science*, **268**, 731–734.
 16. Handa, V., Saha, T. and Usdin, K. (2003) The fragile X syndrome repeats form RNA hairpins that do not activate the interferon-inducible protein kinase, PKR, but are cut by Dicer. *Nucleic Acids Res.*, **31**, 6243–6248.
 17. Zumwalt, M., Ludwig, A., Hagerman, P.J. and Dieckmann, T. (2007) Secondary structure and dynamics of the r(CGG) repeat in the mRNA of the fragile X mental retardation 1 (FMR1) gene. *RNA Biol.*, **4**, 93–100.
 18. Khateb, S., Weisman-Shomer, P., Hershco, I., Loeb, L.A. and Fry, M. (2004) Destabilization of tetraplex structures of the fragile X repeat sequence (CGG)_n is mediated by homolog-conserved domains in three members of the hnRNP family. *Nucleic Acids Res.*, **32**, 4145–4154.
 19. Khateb, S., Weisman-Shomer, P., Hershco-Shani, I., Ludwig, A.L. and 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**, 5775–5788.
 20. Kumari, S., Bugaut, A., Huppert, J.L. and Balasubramanian, S. (2007) An RNA G-quadruplex in the 5' UTR of the NRAS proto-oncogene modulates translation. *Nat. Chem. Biol.*, **3**, 218–221.
 21. Arora, A., Dutkiewicz, M., Scaria, V., Hariharan, M., Maiti, S. and Kurreck, J. (2008) Inhibition of translation in living eukaryotic cells by an RNA G-quadruplex motif. *RNA*, **14**, 1290–1296.
 22. Wieland, M. and Hartig, J.S. (2007) RNA quadruplex-based modulation of gene expression. *Chem. Biol.*, **14**, 757–763.
 23. Han, H., Langley, D.R., Rangan, A. and Hurley, L.H. (2001) Selective interactions of cationic porphyrins with G-quadruplex structures. *J. Am. Chem. Soc.*, **123**, 8902–8913.
 24. Grand, C.L., Han, H., Munoz, R.M., Weitman, S., Von Hoff, D.D., Hurley, L.H. and Bearss, D.J. (2002) The cationic porphyrin TMPyP4 down-regulates c-MYC and human telomerase reverse transcriptase expression and inhibits tumor growth *in vivo*. *Mol. Cancer Ther.*, **1**, 565–573.
 25. Weisman-Shomer, P., Cohen, E., Hershco, I., Khateb, S., Wolfowitz-Barchad, O., Hurley, L.H. and Fry, M. (2003) The cationic porphyrin TMPyP4 destabilizes the tetraplex form of the fragile X syndrome expanded sequence d(CGG)_n. *Nucleic Acids Res.*, **31**, 3963–3970.
 26. Keniry, M.A. (2000) Quadruplex structures in nucleic acids. *Biopolymers*, **56**, 123–146.
 27. Bardin, C. and Leroy, J.L. (2008) The formation pathway of tetramolecular G-quadruplexes. *Nucleic Acids Res.*, **36**, 477–488.
 28. Weisman-Shomer, P., Naot, Y. and Fry, M. (2000) Tetrahelical forms of the fragile X syndrome expanded sequence d(CGG)_n are destabilized by two heterogeneous nuclear ribonucleoprotein-related telomeric DNA-binding proteins. *J. Biol. Chem.*, **275**, 2231–2238.
 29. Weisman-Shomer, P., Cohen, E. and Fry, M. (2002) Distinct domains in the CArG-box binding factor A destabilize tetraplex forms of the fragile X expanded sequence d(CGG)_n. *Nucleic Acids Res.*, **30**, 3672–3681.
 30. Han, F.X., Wheelhouse, T.T. and Hurley, L.H. (1999) Interaction of TMPyP4 and TMPyP2 with quadruplex DNA. Structural basis for the differential effects on telomerase inhibition. *J. Am. Chem. Soc.*, **121**, 3561–3570.
 31. Brown, W.T., Houck, G.E. Jr., Jeziorowska, A., Levinson, F.N., Ding, X., Dobkin, C., Zhong, N., Henderson, J., Brooks, S.S. and Jenkins, E.C. (1993) Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test. *J. Am. Med. Assoc.*, **270**, 1569–1575.
 32. Hagerman, R.J. and Hagerman, P.J. (2002) The fragile X premutation: into the phenotypic fold. *Curr. Opin. Genet. Dev.*, **12**, 278–283.
 33. Jin, P., Zarnescu, D.C., Zhang, F., Pearson, C.E., Lucchesi, J.C., Moses, K. and Warren, S.T. (2003) RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in *Drosophila*. *Neuron*, **39**, 739–747.
 34. Jin, P., Duan, R., Qurashi, A., Qin, Y., Tian, D., Rosser, T.C., Liu, H., Feng, Y. and Warren, S.T. (2007) Pur alpha binds to rCGG repeats and modulates repeat-mediated neurodegeneration in a *Drosophila* model of fragile X tremor/ataxia syndrome. *Neuron*, **55**, 556–564.
 35. Sofola, O.A., Jin, P., Qin, Y., Duan, R., Liu, H., de Haro, M., Nelson, D.L. and Botas, J. (2007) RNA-binding proteins hnRNP A2/B1 and CUGBP1 suppress fragile X CGG premutation repeat-induced neurodegeneration in a *Drosophila* model of FXTAS. *Neuron*, **55**, 565–571.