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

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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
inefficient protein synthesis is the folding of the 3′-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 FMR1 mRNA translation in FX carrier cells can conceivably assuage some of the clinical sequelae of the FMR1 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)porphin (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 FMR1 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 FXSAS. 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)porphin (TMPyP4) was the product of Calbiochem. Its two isomers 5,10,15,20-tetra(N-methyl-3-pyridyl)porphin (TMPyP3) and 5,10,15,20-tetra (N-methyl-2-pyridyl)porphin (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-FMR1-5′-UTR(CGG)ₙ-FL (n = 30 or 99); pCS107: pCS107(CGG)₃₃: pFMR1-5′-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′-3P pCS107(CGG)₃₃ RNA**

Total 1.0μg of BstX1 linearized pCS107(CGG)₃₃ was transcribed in vitro at 37°C for 2h in AmpliScribe SP6 system (Epicenter Biotechnologies) according to the manufacturer’s instructions in a reaction mixture that contained 0.3 μCi [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 nondenaturing 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-FMR1-5′-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-Flashtm 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 DT; 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 x 105 to 5 x 105 cells/well and immediately co-transfected with three plasmids: pFMR1-5’-UTR(CGG)0-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 pFMR1-5’-UTR(CGG)0-FL reporter plasmid. Mixtures with a low ratio of CBF-A encoding vector to reporter plasmid contained 500 ng pFMR1-5’-UTR(CGG)0-FL (n = 0, 30, 99); 0.05 μg pCMV-RL and 2.5 μg pCMV2-Flag CBF-A in 150 μM NaCl. Similarly composed mixtures with a high ratio of CBF-A encoding vector to pFMR1-5’-UTR(CGG)0-FL (n = 0 or 99) reporter plasmid contained 50 ng of pFMR1-5’-UTR(CGG)0-FL (n = 0 or 99) and 2.9 μg pCMV-Flag CBF-A and 5 ng pCMV-RL. Mixtures with a low amount of hnRNAP A2 encoding vector to the reporter plasmid contained 250 ng pFMR1-5’-UTR(CGG)0-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 tosomate 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 pFMR1-5’-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 pFMR1-5’-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 tracts or with a (CGG)99 repeat, the respective average values of RL protein activity were 1404±5360 and 16615±4010 luminescence units and the measured levels of RL RNA, 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 mM [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(TTCTTTGGCGTGAGTT TTCC)-3' or the respective RL forward and reverse
primers; 5'-d(GGGATGAATGGCCTGATATTGAAGAGC)-3' and 5'-d(CAATTTGTACAACGTCAGGTTTAC CACC)-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 pFMRI-5'-UTR(CGG)33-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 horse-radish 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)3 structures in DNA and RNA (25). Here we inquired whether TMPyP4 was also capable of unfolding an intramolecular r(CGG)33 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)33 stretch in mRNA (19) and by tetraplex formations of G-rich tracts in various other mRNA molecules (20–22). We thus...
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-FMR1-5’-UTR(CGG)$_{99}$-FL or pT7-FMR1-5’-UTR(CGG)$_{30}$-FL mRNA was only minimally enhanced in the presence of increasing amounts of TMPyP4. In contrast, the translation of pT7-FMR1-5’-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 hnRNPs A2 and CBF-A on the in vitro 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 translational 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 tetruplex 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 pFMR1-5’-UTR(CGG)$_{99}$-FL or pFMR1-5’-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)$_{99}$-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 FMR1 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
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 pFMR1-5'-UTR(CGG)_{99}-FL or pFMR1-5'-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. 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.

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 pFMR1-5'-UTR(CGG)_{99}-FL | High ratio of agent to pFMR1-5'-UTR(CGG)_{99}-FL |
|---------|-----------------------------------------------|-----------------------------------------------|
|         | FL protein/FL mRNA [N] X-fold increase of translation efficacy | FL protein/FL mRNA [N] X-fold increase of translation efficacy |
|         | FL protein | mRNA | FL protein | mRNA | FL protein | mRNA | FL protein | mRNA |
| TMPyP4  | 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 |
| CBF-A   | 8.8 ± 1.2  | 8.6 ± 1.6 | 1.0 [5] | 1.25 | 5.2 ± 0.7 | 3.1 ± 0.4 | 1.7 [4] | 2.8 |
| TMPyP4 + CBF-A | 14.0 ± 1.6 | 4.5 ± 1.7 | 3.1 [4] | 5.2 |

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 pFMR1-5'-UTR(CGG)_{99}-FL reporter plasmid and that were similarly exposed to TMPyP4, co-transfected with a CBF-A expressing plasmid or left untreated.

5 × 10^5 HEK293 cells were transfected with 500 ng DNA of a pFMR1-5'-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.

5 × 10^5 HEK293 cells were transfected with 50 ng DNA of a pFMR1-5'-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.

N: number of independent experiments.

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 pFMR1-5'-UTR(CGG)_{99}-FL or pFMR1-5'-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 levels ± 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 pFMR1-5'-UTR(CGG)_{99}-FL or pFMR1-5'-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 24 h, 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 efficiencies 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...
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 premutation (CGG)₉₉-FL mRNA (Table 1), we speculated that combining the two agents might increase the translation efficiency of premutation (CGG)₉₀ mRNA. To examine this possibility, matched HEK293 cell cultures were co-transfected with 500 ng of pFMR1-5'-UTR(CGG)₉-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 μM TMPyP4, co-transfected with 2.5 μg 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)₉₀ 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

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 μg 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 μM TMPyP4. (B) Relative translation efficiencies of (CGG)₀-FL mRNA, (CGG)₃₀-FL mRNA or (CGG)₉₀-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 ± SD of the indicated number (N) of independent experiments. (C) Graphic presentation of results tabulated in (B).
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 pFMRI-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 hnRNPA2 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 FMR1 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)$_{5,55–200}$ premutation tract in FMR1 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 hnRNPA2, (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 5'-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 quadruplexes (25).

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quadruplex (19), a formal proof of the nature of the r(CGG)$_{33}$ secondary structure is still lacking. TMPyP$4^-$, but not its positional isomers TMPyP$2^-$ or TMPyP$3^-$ 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 TMPyP$4^-$ or of the quadruplex unfolding proteins CBF-A and hnRNP A2 to increase the efficiency of in vivo translation of premutation (CGG)$_{99}^-$FL mRNA. Our initial results indicated that exposure of HEK293 cells to TMPyP$4^-$, 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 TMPyP$4^-$-induced stimulation was comparable to the enhancement of in vivo translation by CBF-A [Table 1; (20)]. In contrast, lower amounts of TMPyP$4^-$, 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 TMPyP$4^-$ and CBF-A had to be present at an excess over the premutation mRNA.

The required TMPyP$4^-$ 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 TMPyP$4^-$ 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 TMPyP$4^-$ 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 TMPyP$4^-$, CBF-A or hnRNP A2 each by themselves or TMPyP$4^-$ together

Figure 5. Low relative amounts TMPyP$4^-$ 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 µg 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 µM TMPyP$4^-$.
(B) Relative translation efficiencies of 5'UTR (CGG)$_{30}^-$FL mRNA 5'UTR (CGG)$_{99}^-$FL mRNA or 5'UTR (CGG)$_{0}^-$FL mRNA without or in the presence of sub-saturating amount of TMPyP$4^-$ or hnRNP A2 alone or a combination thereof. Displayed are average results ± SD of the indicated number (N) of independent experiments. (C) Graphic presentation of results tabulated in (B).
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) disrupting protein with the premutation repeat tract. In addition to its association with premutation (CGG) mRNP 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.

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