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Effect of *in vitro* promoter methylation and CGG repeat expansion on *FMR-1 expression*

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ABSTRACT

Fragile X syndrome is associated with a CGG repeat expansion in the 5′-untranslated region of the *FMR-1* gene. Within the *FMR-1* promoter a CpG island is frequently methylated in fragile X patients. To identify the effect of methylation on *FMR-1* expression, we transfected methylated and unmethylated constructs containing the *FMR-1* promoter in front of the *CAT* gene (pFXCAT) into COS-1 cells. No difference between methylated and unmethylated DNA was observed initially, whereas reduced *CAT* mRNA levels were observed 48 h post-transfection of the methylated construct and increased CAT activity from unmethylated DNA was observed at 72 h. To determine the effect of a CGG repeat expansion on gene expression, we inserted >200 CGG repeats between the SV40 promoter and the *CAT* gene (pSV2CAT). A 3-fold reduction in CAT activity was observed 24–48 h post-transfection. To study the correlation between CGG repeat expansion and *FMR-1* transcription, we inserted 200 CGG trinucleotide repeats into the pFXCAT construct. Only a slight difference in mRNA levels was found between cells transfected with pFX(CGG)$_{200}$CAT or pFXCAT, but a complete lack of CAT activity was observed with introduction of the repeat. We conclude that a moderate size repeat markedly reduces translation. We propose that the presence of a repeat expansion *per se* is the major factor influencing *FMR-1* function in fragile X syndrome.

INTRODUCTION

Fragile X syndrome is one of the most common forms of inherited mental retardation, with a frequency of 1 in 1250 men and 1 in 2500 females (1). The syndrome is strongly correlated with a fragile site at Xq27.3 and results from loss of activity of the *FMR-1* gene. In many cases fragile X syndrome is due to an expansion of a CGG repeat located in the 5′-untranslated region (5′-UTR) of the *FMR-1* gene (2,3). In the normal population the CGG repeat of the *FMR-1* gene is 2–60 units long. However, individuals with fragile X syndrome show a repeat containing >200 CGG repeats (4–7). Thus, fragile X syndrome is one of a group of disorders associated with trinucleotide repeat expansion (2).

The *FMR-1* gene codes for the FMR-1 protein (FMRP), with possible functions in RNA metabolism or in RNA-containing cellular structures (8,9). RNA binding studies have shown that FMRP is able to bind to its own messenger RNA as well as 4% of human fetal brain mRNA (10). Furthermore, it was recently demonstrated that FMRP interacts with the novel homologues FXR1 and FXR2. The proteins can associate with each other or form homomers (11). Eberhart *et al.* (12) showed that FMRP is a ribonucleoprotein containing both nuclear localization and nuclear export signals. The *FMR-1* gene is expressed at high levels in most, if not all, cells during development, whereas the adult expression pattern is non-uniform, with high levels in organs affected by fragile X syndrome such as brain and testis (13).

Methylation of CpG sequences, preferentially in the promoter regions, is part of the regulation of gene expression and cell differentiation (14,15). In the *FMR-1* promoter region one CpG island is positioned 250 bp upstream of the CGG repeat. This CpG island is frequently found to be methylated in fragile X patients, demonstrating an inverse correlation between DNA methylation and gene expression (16–19). The role of the CGG expansions in establishing the methylation pattern in the CpG island is not known. However, some results indicate that expanded CGG or CCG repeats could display conformational changes which probably induce *de novo* methylation (20). DNA methylation will in this case serve to stabilize the structure and possibly label the repeat for repair (21).

Recent studies of a fragile X syndrome patient suggest that CGG repeat expansion may disturb translation in the absence of methylation (22). It is thus unclear how methylation itself regulates *FMR-1* gene activity and if the methylation frequently observed in fragile X patients is an epiphenomenon or if it has direct effects on *FMR-1* gene function.

In this investigation we asked whether it is the CGG repeat expansion, DNA methylation of the *FMR-1* promoter or a combination thereof that mediates the lowering of gene activity.

MATERIALS AND METHODS

Plasmid construction

The *Xba*I–*Bam*HI fragment from plasmid pE5.1 (David L.Nelson, Baylor College of Medicine, Houston, TX), containing the 5′-end of the *FMR-1* gene, was ligated to the *Xba*I site in the multiple cloning site of pBCAT (Promega, Madison, WI). Both open ends were blunted by DNA polymerase fill-in reaction preceding blunt end ligation. The ligated product was propagated in *Escherichia coli* Hb 101 as pFXCAT (Fig. 1).

A PCR product containing 200 CGG repeats from a male fragile X patient was treated with DNA polymerase 3′ exonuclease activity and blunt end ligated in the *Hin*dIII site of pSV2CAT. For

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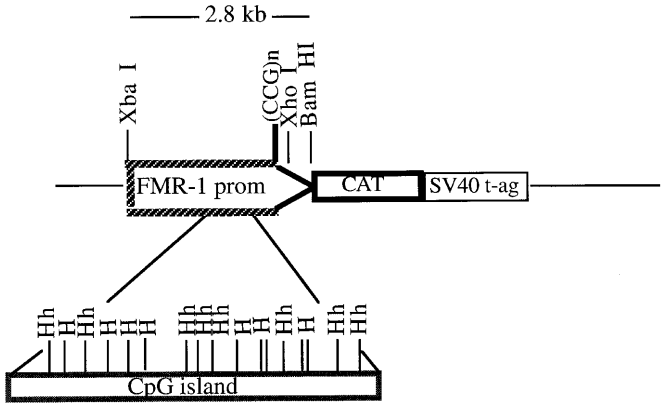

Figure 1. Schematic map of pFXCAT. The positions of the *Hha*I (Hh) and *Hpa*II (H) sites are shown in the CpG island of the FMR-1 promoter. 10 µg pFXCAT was methylated with 15 U M.*Hpa*II in 50 mM Tris–HCl, pH 7.5, 10 mM EDTA, 5 mM 2-mercaptoethanol and 0.2 mM SAM. The methylation efficiency after M.*Hpa*II methylation was always controlled with *Hpa*II and *Msp*I digestion.

construction of pFX$(CGG)_{200}$CAT the CGG repeat product was first cleaved with *Pst*I and *Xho*I and then inserted in the pFXCAT construct. For PCR amplification of the CGG repeat expansion Dynazyme tbr DNA polymerase (Finnzymes OY, Finland) was used with upstream primer (primer 1) TGCAGAAATG GGCGTTCTGG and downstream primer (primer 2) GCCCTAGAGC CAAGTACCTT GT. An aliquot of 0.1 µg genomic DNA was added to a solution of 10% DMSO, 0.20 mM dNTP, 10× Dynazyme buffer containing 2.5 mM $MgCl_2$, 2 U Dynazyme and 6 µM of each primer. The amplification profile was as follows: 94°C for 4 min, four cycles of 94°C for 1 min, 57°C for 2 min, 72°C for 3 min and 31 cycles of 94°C for 35 s, 57°C for 40 s, 72°C for 2.30 min. The PCR product was purified after agarose gel electrophoresis using DNA Purification Kit Prep-A-Gene® (BioRad, Hercules, CA) before further treatment.

In vitro methylation

An aliquot of 10 µg plasmid DNA was incubated with *Hpa*II methyltransferase (Fermentas, Lithuania) overnight at 37°C in a reaction mixture containing 50 mM Tris–HCl, pH 7.5, 300 mM *S*-adenosylmethionine (SAM), 10 mM EDTA and 5 mM dithiothreitol (DTT). The reaction was terminated by adding 200 µg/ml proteinase K for 1 h at 37°C followed by phenol extraction and chloroform/isoamyl alcohol (24:1). The DNA was precipitated with ethanol. The efficiency of methylation was controlled by digestion of methylated pFXCAT with *Hpa*II and *Msp*I. Prior to transfection the methylated DNA was cut with *Hpa*II and purified by agarose gel electrophoresis.

Cell culture and transfection

COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithesburg, MD) supplemented with 10% fetal calf serum and 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Samples of 1×10^6 cells were transfected with 2 µg supercoiled methylated or unmethylated pFXCAT DNA. Electroporation was used to transfect cells that were pulsed at 1050 µF, 220 V with a pulse time of ~25 ms.

CAT assay

To control the activity of the *CAT* gene, the recipient cells were harvested at different time points after transfection. The cells were washed with PBS and scraped off with a rubber policeman. Total cell extracts were transferred to Eppendorf tubes filled with 150 µl 0.25 M Tris–HCl, pH 7.8, sonicated and incubated at 60°C for 10 min. After centrifugation for 10 min the supernatant was used for enzymatic reaction with [^{14}C]chloramphenicol and acetyl-CoA.

To each tube 20 µl acetyl-CoA (4 mM) and 2 µl 200 µCi [^{14}C]chloramphenicol were added. The mixture was incubated for 17 h at 37°C. After incubation chloramphenicol and its derivatives were extracted with ethyl acetate and the acetylated chloramphenicol (AC) and the non-acetylated form were separated on thin layer silica gel plates (Merck, Darmstadt, Germany). The plates were developed in chloroform/methanol (95:5) and then autoradiographed (23). The percentage conversion of [^{14}C]chloramphenicol to the acetylated form (AC) was quantified using a Fuji Bas 1000 IP Reader (Fuji, Japan).

RT–PCR

For first strand cDNA synthesis 1 µg total RNA from transfected cells (24) dissolved in DEPC-treated water was used with a Ready To Go kit (Pharmacia Biotech, Uppsala, Sweden) containing M-MuLV reverse transcriptase and an oligo(dT) primer to generate first strand cDNA. The RNA sample was denatured and placed at 37°C for 60 min. The complete first strand reaction was heat inactivated at 90°C for 5 min. An aliquot of 5 µl first strand reaction was used for PCR amplification. Amplifications were initially carried out using the primers (CAT-1) GAGGGCATTTCAGTCAGTTGC and (CAT-2) TGAAACTCACCCAGGGATTG, corresponding to nucleotides 4985–5007 and 5359–5378 of the *CAT* gene. The reaction contained 10 µl 10× Dynazyme buffer,1 µl 20 mM dNTP mix, 30 pmol upstream primer (CAT-1), 30 pmol downstream primer (CAT-2) and water to 100 µl and 2.5 U Dynazyme enzyme. The PCR amplification profile was 94°C for 4 min, four cycles of 94°C for 1 min, 57°C for 2 min, 72°C for 3 min and 31 cycles of 94°C for 35 s, 57°C for 40 s, 72°C for 2.30 min. The PCR products were monitored on a 1% agarose gel.

RESULTS

The purpose of this study was to determine the effect of *in vitro* methylation of the *FMR-1* promoter region and insertion of a CGG repeat expansion in the 5′-UTR of *FMR-1* on gene expression in cell culture. We therefore constructed a plasmid containing the *FMR-1* promoter and the transcription start and exon 1 from *FMR-1* in front of the *CAT* gene and SV40 small t antigen (pFXCAT; Fig. 1).

In vitro methylation

To change the methylation state of this construct, we incubated the pFXCAT construct with the methyltransferase M.*Hpa*II so that the internal cytosine in the CCGG target sequence was methylated (the positions of the nine *Hpa*II sites in the CpG island are indicated in Figure 1). C^{5m}CGG methylation inhibits *Hpa*II but not *Msp*I digestion. Both enzymes were used to control for methylation efficiency. In order to assess the effect of methylation on gene expression, COS-1 cells were transfected with the modified DNA. Prior to transfection the methylated DNA was digested with *Hpa*II, separated by gel electrophoresis and purified to

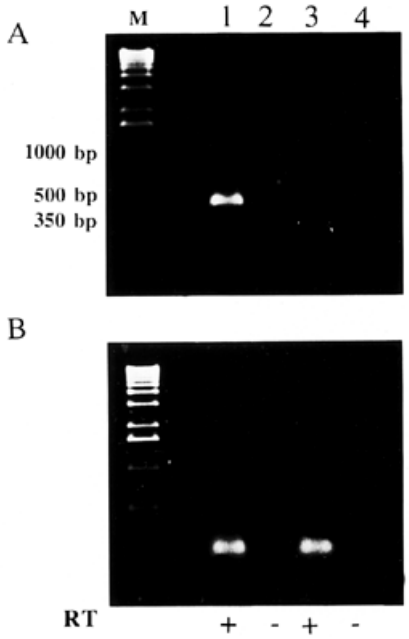

Figure 2. RT–PCR from total RNA prepared from pFXCAT-transfected COS-1 cells 48 h after DNA transfer. The RNA was treated with DNase I before RT. (**A**) Lane 1, pFXCAT with RT+; lane 2, pFXCAT without RT–; lane 3, pFXCAT methylated with *Hpa*II with RT+; lane 4, pFXCAT methylated without RT–. M, a 1 kb ladder from Promega was used as marker. (**B**) Primers for the housekeeping gene GAPDH were used as a control.

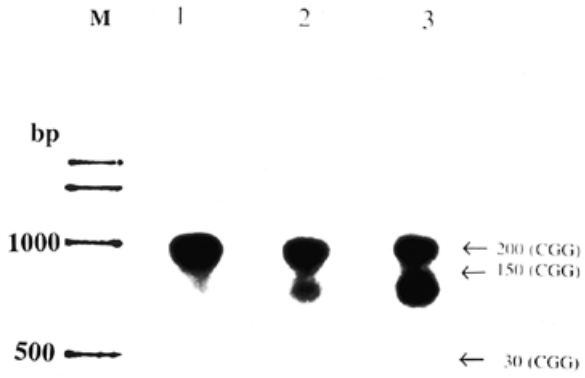

Figure 3. Southern blot analysis of PCR amplification of the CGG repeat region with different amounts of genomic DNA from a male fragile X patient. Lane 1, 50 ng; lane 2, 75 ng; lane 3, 100 ng DNA. The blot was hybridized with the ^{32}P-labeled *Bcl*I–*Xho*I fragment from pFXCAT.

Table 1. CAT enzyme activity was measured 24, 48 and 72 h after transfection of 2 µg DNA/10^6 COS-1 cells, using the electroporation method (1050 µF, 220 V)

Construct	Acetylation (%)		
	24 h	48 h	72 h
pFXCAT	2	3	10
pFXCAT-CH_3	2	3	3
pSV2CAT	42	61	94
pBCAT	0.5	0.5	1

Percentage acetylation was measured as acetylated [^{14}C]chloramphenicol divided by the total amount added. pFXCAT methylated with *Hpa*II methyltransferase prior to transfection is indicated as pFXCAT-CH_3. pSV2CAT was used as positive control. pBCAT was used as a control for basal transcription level. Data are means for three separate transfection experiments.

exclude contamination with unmethylated DNA. Recipient cells were harvested at various intervals after transfection. *In vitro* methylation of the *FMR-1* promoter did not change the CAT activity of pFXCAT during the first 48 h after gene transfer (Table 1) and the activity was similar to that after transfection of non-methylated DNA. At 72 h after transfection a 3-fold increase in CAT activity was observed with the unmethylated contruct, whereas methylated pFXCAT remained at low levels (Table 1). The level of *CAT* mRNA was determined by RT-PCR of RNA prepared from cells 48 h after transfection of methylated and unmethylated pFXCAT. No *CAT* mRNA was detected in cells transfected with methylated pFXCAT, however RNA isolated from cells transfected with unmethylated pFXCAT resulted in a 396 bp fragment after RT–PCR (Fig. 2).

CGG repeat expansion and *FMR-1* promoter activity

The CGG repeat expansion in the first exon of the *FMR-1* gene is assumed to have an important role in development of the fragile X phenotype (3–5,7,16). To characterize the effect of a CGG repeat expansion (≥200) on gene expression in general, we PCR amplified a DNA fragment from a male fragile X patient with an expanded repeat (Fig. 3). PCR amplification of this ~1000 bp sequence containing 100% GC was non-trivial and different conditions were therefore evaluated. The PCR amplification resulted in a product of 1000 bp containing ~200 CGG repeats (Fig. 3). The CGG_{200} sequence was then blunt ended by a DNA polymerase fill-in reaction and inserted in construct pSV2CAT (Fig. 4A). Plasmid pSV2CAT contains the early SV40 promoter controlling the *CAT* gene and SV40 poly(A) signal.

To determine the biological activity, COS-1 cells were transfected with pSV2CAT or pSV2$(CGG)_{200}$CAT. The CAT activity was reduced to <20% acetylation with pSV2$(CGG)_{200}$CAT, whereas transfection with pSV2CAT resulted in 80% acetylation at 24 and 48 h post-transfection (Fig. 5).

To examine the effect of a CGG repeat expansion on *FMR-1* promoter activity, plasmid pFXCAT was modified with an insert of the expanded CGG PCR product after *Pst*I and *Xho*I digestion (Fig. 4B). This plasmid, pFX$(CGG)_{200}$CAT, with the expanded repeat in the 5′-UTR of exon 1 of the *FMR-1* gene, was used to analyze the result of *FMR-1* CGG repeat expansion *in vitro*.

In order to determine the biological activity of pFX$(CGG)_{200}$-CAT, we transfected methylated and unmethylated plasmid DNA into COS-1 cells. The recipient cells were harvested after 24, 48 and 72 h. Both the unmethylated and the methylated construct completely lacked all CAT activity (data not shown).

To assess the effect of CGG repeat expansion on transcriptional activity, we transfected COS-1 cells with unmethylated pFX$(CGG)_{200}$CAT and analysed mRNA levels by RT–PCR. To study the normal rate of transcription the pFXCAT plasmid was used as a control. Only a slight difference in transcriptional

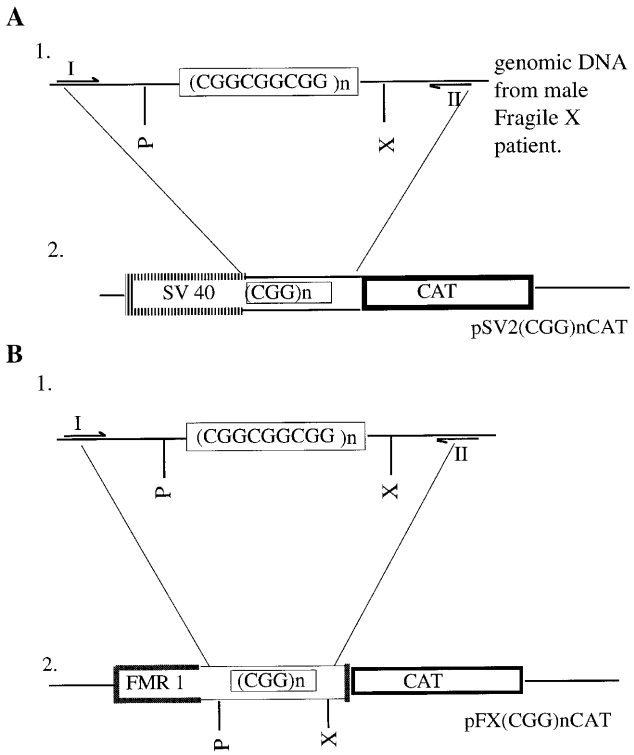

Figure 4. Schematic description of the insert of a PCR amplified CGG repeat fragment from a fragile X patient in pSV2CAT (**A**) and pFXCAT (**B**). H, *Hin*dIII; P, *Pst*I; X, *Xho*I.

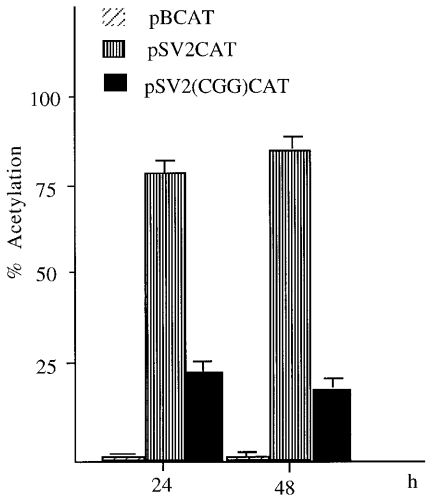

Figure 5. CAT activity 24 and 48 h post-transfection in COS-1 cells transfected with pBCAT, pSV2CAT and pSV2(CGG)$_{200}$ CAT. pBCAT (Promega) contains only the *CAT* gene and SV40 poly(A) signal and serves as a control for non-specific acetylation.

activity was observed at 48 h between cells transfected with pFX(CGG)$_{200}$CAT or pFXCAT (Fig. 6).

DISCUSSION

Loss of *FMR-1* expression is widely accepted to be the cause of fragile X syndrome. It is, however, controversial whether this loss

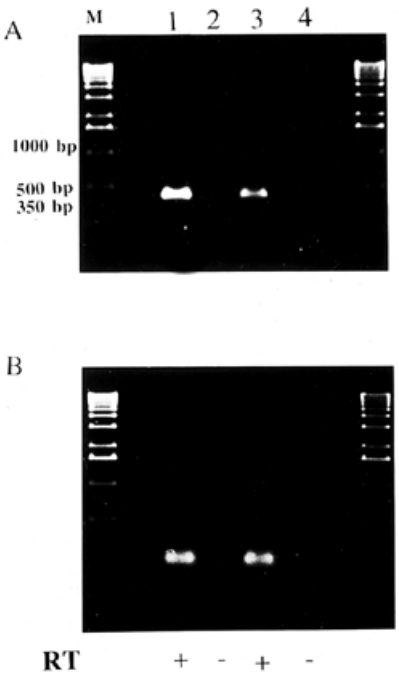

Figure 6. (**A**) RT–PCR from total RNA prepared 48 h after transfection of pFXCAT (lane 1, +RT; lane 2, –RT) and pFX(CGG)$_{200}$CAT (lane 3, +RT; lane 4, –RT) in COS-1 cells. Note equal intensity of bands from constructs with or without the repeat expansion. M, a 1 kb ladder was used as marker. (**B**) Primers for GAPDH were used to control for RNA quality.

is the result of a CGG expansion *per se* or commonly occurring hypermethylation of the promoter region (22). Furthermore, the role of CpG methylation of the CpG island in the *FMR-1* promoter in regulating *FMR-1* expression is still not clear. Recently it was demonstrated that methylation of all CpG dinucleotides by *Sss*I methylase blocked *FMR-1* promoter activity 48 h after transfection in the absence of a repeat expansion (27). On the other hand, a fragile X patient with a lung tumour had *FMR-1*-expressing tumor cells despite methylation of both the *Eag*I and *Bss*HII sites in the *FMR-1* promoter (30). It is possible that a special CpG site has to be methylated to reduce gene activity. Such CpG sites have previously been identified (29) and may apply also to regulation of the *FMR-1* gene. Novel methylation sites may in fact be produced by the expansion itself and it has been demonstrated that DNA methyltransferase can recognize and selectively methylate unusual DNA structures like trinucleotide CGG repeats *in vitro* (19). Furthermore, d(CGG)$_n$ oligonucleotides form a complex structure with properties similar to tetrahelical DNA under physiological conditions and 5-methylation of cytosine stabilizes this structure (21).

We have studied methylation of the *FMR-1* promoter and the effect of a CGG repeat expansion in the 5′-UTR *in vitro*. The *FMR-1* promoter is a relatively weak inducer of CAT activity in COS-1 cells, despite the fact that these cells have been shown to be among the best cell lines for *FMR-1* expression (31). Methylation *per se* did not inhibit transient expression of plasmid pFXCAT-CH$_3$ in COS-1 cells during the first 48 h after transfection, indicating that methylation may be less related to the loss of expression seen in patients. However, we did observe reduced transcription in cells transfected with pFXCAT-CH$_3$ 48 h after DNA transfer, indicating that methylation may down-regulate

the *FMR-1* gene. This was further supported by the increase in CAT activity seen using unmethylated pFXCAT in contrast to methylated DNA 72 h post-transfection. The delayed difference in expression may also be due to chromatin assembly of the *trans*-DNA in the nucleus. It is well known that microinjected supercoiled plasmid DNA is assembled into minichromosomes 48–72 h after gene transfer (26). This may lead to more efficient inhibition of the methylated construct due to interaction between methyl groups and histone octamers, histone 1 or other methyl CpG binding protein. Stability of the CAT enzyme in the cell might maintain the base level of gene activity seen in the cells transfected with methylated pFXCAT even in the absence of *de novo* transcription.

The biological activity of pSV2$(CGG)_{200}$CAT was reduced as early as 24 h after gene transfer and complete loss of CAT activity was observed after transfection of pFX$(CGG)_{200}$CAT. These findings taken together support the notion that a CGG repeat expansion alone can inhibit gene expression (22). The presence of similar transcription levels in cells expressing pFX$(CGG)_{200}$CAT and pFXCAT implies that translation rather than transcription is influenced by the change in the number of CGG repeat units, at least in the short size range of pathological $(CGG)_n$ expansions. These results are consistent with observations made recently using fibroblast subclones from a mildly affected fragile X patient (22).

In conclusion, we have demonstrated that a CGG expansion as well as *in vitro* methylation of the *FMR-1* promoter regulate gene expression. The CGG repeat expansion affects translation but not transcription and acts independently of methylation. We propose that the repeat expansion *per se* is a primary factor determining *FMR-1* gene expression, possibly modulated by methylation events. Future studies using cell lines transfected with pFX$(CGG)_n$CAT containing various repeat unit sizes may reveal in what way the CGG repeat expansion itself can influence the pattern of methylation of flanking CpG sequences.

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REFERENCES

1 Nussbaum,R.L. and Ledbetter,D.H. (1986) *Annu. Rev. Genet.*, **20**, 109.
2 Lindblad,K. and Schalling,M. (1996) *Acta Padiat.*, **85**, 265–271.
3 Verkerk,A.J.M.H., Pieretti,M., Sutcliffe,J.S., Fu,Y.H., Kuhl,D.P.A., Pizzuti,A. and Reiner,O. (1991) *Cell*, **65**, 905–914.
4 Fu,Y.H., Kuhl,D., Pizzuti,A., Pieretti,M., Sutcliffe,J.S., Richards,S., Verkerk,A., Holden,J., Fenwick,R.J., Warren,S.T., Oostra,B.A., Nelson,D.L. and Caskey,C.T. (1991) *Cell*, **67**, 1047–1058.
5 Kremer,E.J., Pritchard,M., Lynch,M., Yu,S., Holman,K., Baker,E., Warren,S.T., Schlesinger,D., Sutherland,G.R., Richards,R.I. (1991) *Science*, **252**, 1711–1714.
6 Rousseau,F., Heitz,D., Biancalana,V., Blumenfeld,S., Kretz,C., Boué,J., Tommerup,N., Van der Hagen,C., DeLozier-Blanchet,C., Croquette,M.F., Gilgenkrantz,S., Jalbert,P., Voelckel,M.A., Oberlé,I. and Mandel,J.L. (1991) *N. Engl. J. Med.*, **325**, 1673–1681.
7 Yu,S., Mulley,J., Loesch,D., Turner,G., Donnely,A., Gedeon,G., Hillen,D., Kremer,E., Lynch,M., Pritchard,M., Sutherland,G.R. and Richards,R.I. (1992) *Am. J Hum. Genet.*, **50**, 968–980.
8 Ashley,C.T., Sutcliffe,J.S., Kunst,C.B., Leiner,H.A., Eichler,E.E., Nelson,D.L. and Warren, S.T. (1993) *Nature Genet.*, **4**, 244–251.
9 Siomi,H., Matunis,M.J., Nussbaum,R.L. and Dreyfuss,G. (1993) *Cell*, **74**, 291–298.
10 Gibson,T.J., Thompson,J.D. and Heringa,J. (1993) *FEBS Lett.*, **324**, 361–366.
11 Zhang,Y., O'Connor,J.P., Siomi,M.C., Srinivasan,S., Dutra,A., Nussbaum,R.L. and Dreyfuss,G. (1995) *EMBO J.*, **14**, 5358–5366.
12 Eberhart,D.E., Malter,H.E., Feng,Y. and Warren,S. (1996) *Hum. Mol. Genet.*, **5**, 1083–1091.
13 Hinds,H.L., Ashley,C.T., Sutcliffe,J.S., Nelson,D.L., Warren, S.T., Housman,D.E. and Schalling,M. (1993) *Nature Genet.*, **3**, 36–43.
14 Doerfler,W. (1983) *Annu. Rev. Biochem.*, **52**, 93–124.
15 Cedar,H. (1988) *Cell*, **53**, 3–4.
16 Oberlé,I., Rousseau,F., Heitz,D., Kretz,C., Devys,D., Hanauer,A., Boué,J., Bertheas,M.F. and Mandel,J.L. (1991) *Science*, **252**, 1097–1102.
17 Bell,M.V., Hirst,M.C., Nakahori,Y., MacKinnon,R.N., Roche,A., Flint,T.J., Jacobs,P.A., Tommerup,N., Tranebjaerg,L., Froster,I.U., Kerr,B., Turner,G., Lindenbaum,R.H., Winter,R., Pembrey,M., Thibodeau,S. and Davies,K.E. (1991) *Cell*, **64**, 861–866.
18 Hansen,R.S., Gartler,S.M., Scott,C.R., Chen,S., Laird,C.D. (1992) *Hum. Mol. Genet.*, **1**, 571–578.
19 Pieretti,M., Zhang,F., Fu,Y.H., Warren,S.T., Oostra,B.A., Caskey,C.T. and Nelson,D.L. (1991) *Cell*, **66**, 817–822.
20 Smith,S.S., Laayoun,A., Lingeman,R.G., Baker,D.J. and Riley,J. (1994) *J. Mol. Biol.*, **243**, 143–151.
21 Fry,M. and Loeb,L.A. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 763–767.
22 Feng,Y., Zhang,F., Lokey,L.K., Chastain,J.L., Lakkis,L. Eberhart,D. and Warren, S.T. (1995) *Science*, **268**, 731–734.
23 Gorman,C.M., Moffat,L.F. and Howard,B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
24 Puisant,C. and Houdebine,L.M. (1990) *BioTechniques*, **8**, 148–149.
25 Heitz,D., Devys,D., Imbert,G., Kretz,C. and Mandel,J.L. (1992) *J. Med. Genet.*, **29**, 794–801.
26 Buschausen,G., Wittig,B., Gräßmann,M. and Gräßmann,A. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 1177–1181.
27 Hwu,W.-L., Lee,Y.-M. and Wang,T.-R. (1993) *Biochem. Biophys. Res. Commun.*, **193**, 324–329.
28 Bird,A.P. (1987) *Nature*, **321**, 209–213.
29 Wölfl,S., Scrader,M. and Wittig,B. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 271–275.
30 De Graaf,E., Willemsen,R., Zhong,N., De Die-Smulders,C.E.M., Ted Brown,W., Freling,G. and Oostra,B. (1995) *Am. J. Hum. Genet.*, **57**, 609–618.
31 Devys,D., Lutz,Y., Rouyer,N., Bellocq,J.-P. and Mandel,J.-L (1993) *Nature Genet.*, **4**, 335–341.