Original Paper

European Journal of Human Genetics

Eur J Hum Genet 1997;5:293–298

Received: May 5, 1997
Revision received: June 20, 1997
Accepted: June 23, 1997

FMR1 Premutation Allele $(CGG)_{81}$ Is Stable in Mice

Carola J.M. Bontekoe
Esther de Graaff
Ingeborg M. Nieuwenhuizen
Rob Willemsen
Ben A. Oostra

MGC Department of Clinical Genetics,
Erasmus University,
Rotterdam, The Netherlands

Key Words
Fragile X syndrome
FMR1 gene
Premutation
Repeat stability
Transgenic mouse

Abstract
Fragile X syndrome is caused by an expansion of the CGG repeat present in the 5′ UTR of the *FMR1* gene. A lot has been elucidated about the genetics of the disease, but not much is known about the mechanisms involved in repeat instability. Transgenic animals with a premutation allele [$(CGG)_{11}AGG(CGG)_{60}CAG(CGG)_8$] in the human *FMR1* promoter were generated to study the inheritance of this repeat in mice. Three independent lines, B6, B7 and B29, in total 263 transgenic animals, were tested for repeat instability. In all meiosis and mitosis tested, the repeat inherited stably. This suggests that other factors might be important in repeat (in)stability.

Introduction

Since 1991, more than 12 diseases have been identified that are caused by amplifications of trinucleotide repeats. Although we have learned a lot about the genetics of the 'trinucleotide repeat diseases', not much is known about the mechanism of repeat expansion. The fragile X syndrome, one of these trinucleotide repeat diseases, is an X-linked disorder affecting 1 in 4,000 males and 1 in 6,000 females [1]. The main characteristics of the syndrome are mental retardation and macroorchidism [2]. The trinucleotide repeat causing the disease is a CGG repeat present in the 5′ untranslated region (UTR) of the fragile X mental retardation 1 gene *(FMR1)* [3]. Expansions of this repeat above 200 triplets, full mutations, cause methylation of the CpG island including the promoter region and the repeat itself [4, 5]. This methylation blocks the transcription and therefore results in the lack of the fragile X mental retardation protein (FMRP) [6, 7].

In nonaffected individuals, the highly polymorphic CGG repeat varies between 6 and 200 triplets with 29–32 triplets being the most common. These alleles are divided into two classes. Normal alleles, repeats between 6 and 54 triplets, inherited stably, whereas so-called premutation alleles of 43 to 200 repeat units show instability upon transmission [8, 9]. These premutations do not cause the disease, but they are prone to expand to full mutations in next generations.

Instable inheritance of the CGG repeat is observed via paternal and maternal transmission, but full mutations only arise upon female transmission. Inheritance via the paternal line never gives rise to full mutation alleles. Because of this parent of origin effect it has been suggested that expansions occur during gametogenesis [10]. However, it is also possible that expansions occur during early embryonic development. For obvious reasons, data about oocytes and early embryogenesis are limited. Recently, female fetuses showing a full mutation in somatic tissues were described whereas full mutations were alrea-

KARGER
E-Mail karger@karger.ch
Fax + 41 61 306 12 34
http://www.karger.ch

1018–4813/97/0055–0293$15.00/0

This article is also accessible online at:
http://BioMedNet.com/karger

Dr. Ben A. Oostra
Department of Clinical Genetics
Erasmus University, PO Box 1738
NL–3000 DR Rotterdam (The Netherlands)
Tel. 31 10 4087198, fax 31 10 4087200, e-mail oostra@kgen.fgg.eur.nl

dy present in the oocytes [10]. Still the exact timing of the repeat expansion is not known, but it shows that the female germline is not protected against full mutation expansion.

In sperm of patients only permutations were found [11], suggesting contraction of full mutations in the immature testis. A hypothesis is that during spermatogenesis a kind of selection mechanism prevents germline precursors with a full mutation to arise or to develop into mature spermatocytes [10]. It has been suggested that FMRP is necessary for gametogenesis although the knock-out mice for *Fmr1*, lacking Fmrp completely, are fertile [12]. Previously, transmission of a deletion causing fragile X syndrome through a family could be traced back to the deceased grandfather. This grandfather transmitted the deletion to 3 daughters indicating that the deletion was already present in his sperm cells [13]. These findings make it less likely that FMRP is necessary to produce spermatocytes.

Moreover, somatic instability does also occur. Most patients show different lengths of full mutations often present as a smear on Southern blot analysis [14]. This shows that repeat length changes still occur in the early embryo. It has been estimated that in 40% of the fragile X patients a premutation is also present [15]. These mosaics show always one premutation allele and a smear for the full mutation. From previous studies, it is clear that this mosaic pattern is not caused by permanent mitotic instability. It is thought that mitotic instability can arise during a fixed window in early embryogenesis [16–18].

At this moment it is not known which mechanisms are involved in expansions, mitotic or meiotic. To study these mechanisms, an animal model is required. Only in an animal model it will be possible to study gametogenesis and early embryogenesis at specific time points. To study the behavior of a premutation allele upon next generations, we generated transgenic mice with a premutation allele, $(CGG)_{81}$ in the *FMR1* promoter. The *FMR1* promoter was fused to the reporter gene *LacZ*. A similar construct with a $(CGG)_{16}$ repeat showed a similar expression in the complete *FMR1* gene [19]. Assuming that the mouse model resembles the human situation regarding repeat instability, expansion of the repeat from a premutation to a full mutation is expected to be followed by methylation of the promoter region of *FMR1* [5, 20]. It is therefore expected that if the repeat in these transgenic mice expands to a full mutation, expression of LacZ will be absent due to methylation of the *FmR1* promoter. Using this fusion gene as a transgene, the instability of the repeat upon transmission was studied.

Materials and Methods

Construct with the Expanded CGG Repeat

The construct of the *FMR1* promoter fused to the *LacZ* reporter *(FMR1/LacZ)* was kindly provided by Hergersberg et al. [19]. The *Nru*I/*Xho*I fragment containing a repeat of 16 CGGs in this construct was replaced by a *Nru*I/*Xho*I fragment containing a premutation allele, present in pRN2 [21]. The repeat present in this fragment was amplified from a premutation carrier $(CGG)_{81}$, $[(CGG)_{11}AGG(CGG)_{60}CAG(CGG)_8]$. This repeat contains a pure $(CGG)_{60}$ tract. This length is sufficient to cause instability in humans. Downstream of the pure $(CGG)_{60}$ repeat, there is a CAG interruption and a $(CGG)_8$.

Transgenic Mice

The transgene was isolated from agarose gel after a *Not*I digest. For microinjection, a transgene concentration of 5 ng/µl was used. The transgene was injected in the pronuclei of fertilized oocytes of FVB mice. After microinjection, the oocytes were transferred to a BCBA foster.

Transgenic animals were identified with PCR performed at tail DNA. PCR conditions were 20″ 94 °C, 20″ 65 °C and 45″ 72 °C and primers E (5′ TGGGCCTCGAGCGCCCGCAGCCCACCTCTC 3′) and AGβ (5′ GGGATGTGCTGCAAGGCGATTTAAG 3′) were used. The product was visualized on a 2% agarose gel.

Repeat Stability

To determine the repeat length in the transgenic animals, radioactive PCR was performed with primers C (5′ GCTCAGCTCCGTTTCGGTTTCACTTCCGGT 3′) and ACβ (5′ GTACCCGGGGATCCTCTAGCGCCGGGA 3′). PCR conditions were as described by Deelen et al. [22]. The PCR products were run on a polyacrylamide gel. Small changes in repeat length could be detected in this way. To determine whether there were large expansions of the repeat, Southern blot analysis was performed for a number of transgenic animals.

Results

Transgenic Animals with Enlarged CGG Repeat

Unstable CGG repeats in humans of a premutation size have a length between 43 and 200 repeat units. To introduce a repeat length in this range into mouse, we have replaced the normal CGG repeat in the *FMR1* promoter by a repeat of 81 repeat units. The *FMR1* promoter was located upstream a *LacZ* reporter gene (fig. 1).

Transgenic mice were obtained by microinjection of the construct pENPL2 into fertilized eggs. Tail DNA from pups was tested for the presence of the transgene and the number of CGG repeats. Three independent transgenic lines, B6, B7 and B29, were obtained. The copy number of the transgene was one for B7 and B29 and two for B6. This was determined by Southern blot analysis (data not shown). The repeat length in the founder mice was exactly 81 repeat units as in the original DNA construct used for

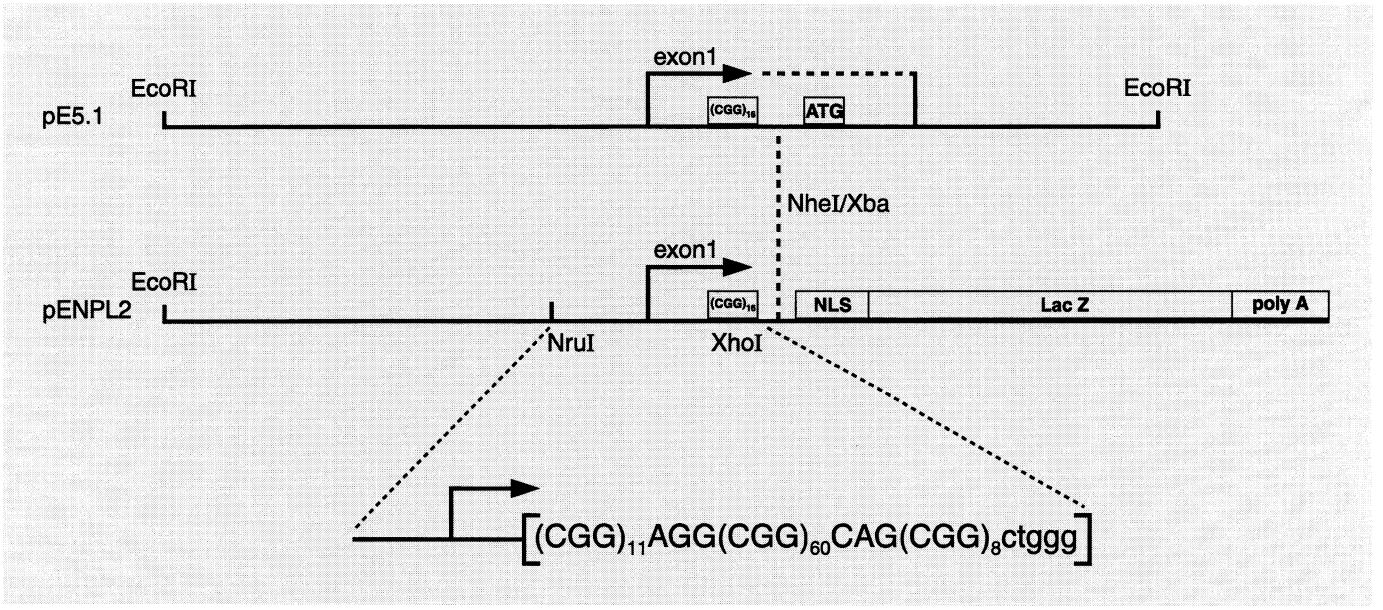

Fig. 1. Schematic representation of pENPL2 containing the *FMR1* promoter region with $(CGG)_{81}$ repeat upstream of the *LacZ* reporter gene.

microinjection. In order to study the stability of the repeat upon germline transmission, the transgenic founders were crossed with wild-type FVB mice. Transgenics of the first generation were crossed with transgenic litter mates if possible. When there were no transgenic litter mates available, transgenic animals were crossed with wild-type FVBs. Line B6 and B7 were crossed till the seventh generation, line B29 was crossed till the sixth generation. For each line, the transgene is situated on one of the autosomes as was concluded from the ratio of male and female transgenic progeny.

In total, 263 transgenic animals were identified as shown in table 1. Because of the parent of origin effect for the repeat instability seen in fragile X syndrome, the sex of the transmitting parent is depicted. In total, 25 times the repeat was transmitted by a male and 25 times by a female. When transgenic litter mates were crossed, we scored them for both male and female transmission. In this way 101 transgenic males and 98 transgenic females were generated.

In addition, the homozygous transgenic mice from the last generation were crossed with wild-type mice. For each line, 1 female and 1 male mouse is crossed with a wild-type animal. The next generation shows 64 heterozygous mice, 35 males and 29 females.

Table 1. Transgenic offspring of transgenic mouse strains

	Transgenic lines			
	B6	B7	B29	total
Male transmission	9	8	8	25
Female transmission	8	10	7	25
Transgenic male offspring	31	42	28	101
Transgenic female offspring	36	34	28	98
F1 males[1]	11	9	15	35
F1 females[1]	10	9	10	29
Transgenic offspring total	88	94	81	263

[1] Offspring of a cross between a male/female homozygous transgenic mouse and a control mouse.

Repeat Stability

For all transgenic animals, radioactive PCR was performed to score for possible (small) differences in repeat length. All transgenic animals showed exactly the same size of PCR product, indicating that the repeat inherited stably (fig. 2). Southern blot analysis was performed on a number of samples to detect possible larger expansions especially when the radioactive PCR failed or gave a weak signal. Also by Southern blot analysis, no different repeat

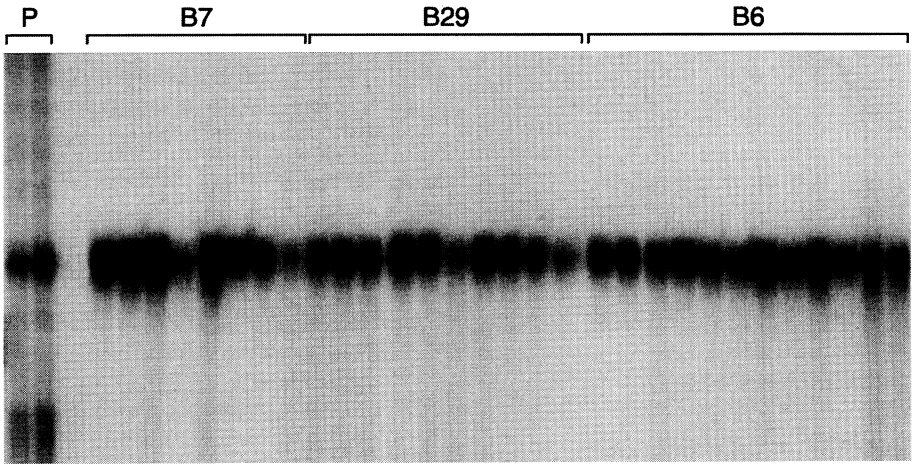

Fig. 2. The size of the (CGG) in the three independent transgenic lines was determined by PCR analysis. P shows the size of the (CGG) repeat in the original plasmid: $(CGG)_{81}$.

length could be identified indicating that the $(CGG)_{81}$ repeat inherited stably in all mitosis and meiosis tested. LacZ expression in testes was tested on the three different strains. LacZ expression was found in testes of the B7 line. Strain B29 failed to show expression due to a deletion in the LacZ gene in the transgenic mice. Strain B6 did not show LacZ expression for unknown reasons. No methylation of the *FMR1* promoter region was detected in this transgenic strain (data not shown).

Discussion

In this study we describe the inheritance of a CGG repeat of 81 repeat units in the human *FMR1* promoter when present as a transgene. Expanded repeats of this size show instable inheritance in humans upon transmission. To our knowledge, instability of the CGG repeat in mice has not been observed. We used a repeat length of 81 repeat units. The longest pure CGG tract within this repeat is 60 CGGs. This number has shown to be sufficient to cause instability in humans [23]. The transgenic mice for the CGG repeat showed stable inheritance in all mitosis and meiosis studied, regardless of the sex of the transmitting parent. There might be a number of explanations for this.

In the murine *Fmr1* CGG repeats of 9–12 repeat units have been identified in different mice strains. The size of the repeat necessary to cause instability in mice is not known. It is not known whether CGG repeat instability in mice exists. It might be a human-specific phenomenon. We assumed that underlying mechanisms causing instability would be present in humans and mice. Transgenic mice containing an expanded repeat in the androgen receptor cDNA, the Huntington cDNA or the SCA1 cDNA showed stable inheritance through many meiosis [24–26], but recently, studies for other trinucleotide diseases, such as Huntington and myotonic dystrophy, have shown that small changes in repeat length can occur [27–29].

Expansions of the CGG repeat in humans are only found at the 3′ end of the repeat [23, 30]. The observation of AGG triplets interrupting the CGG tract has led to the suggestion that these interruptions provide stability to the repeat [23, 31]. Likewise, instability might be the result from loss of AGG interruptions or from the growth of the repeat at the 3′ end. It has been proposed that repeats with an uninterrupted tract of 34 triplets are becoming unstable [23]. The CGG repeat in our construct contains a pure CGG tract of 60 repeat units, and therefore this length should be sufficient to cause instability. Downstream of the pure $(CGG)_{60}$ tract a CAG triplet is found followed by a tract of 8 CGG repeats. This CAG interruption, although present in some mammals [32], has never been detected in humans. It is therefore likely that this CAG triplet originated from the cloning procedures, either in the *Escherichia coli* bacteria or as a PCR artifact. It is possible that this CAG interruption, like AGG interruptions in humans, has a stabilizing effect on the repeat.

The site of integration of the transgene might determine the stability of the repeat. Sequences in the interrupted chromosomal region might influence the behavior of the transgene. The *FMR1* gene is normally present at the X-chromosome, while the transgene was found to be present at one of the autosomes. Since we do not know which mechanisms are involved in CGG expansions, the

X chromosome might play an important role. Possibly mechanisms involved in X-inactivation might play a role in the stability of the repeat. Futhermore, flanking sequences of the *FMR1* promoter might be important in generating instability. The region we used as promoter is sufficient to allow normal gene expression as described by Hergersberg et al. [19] for the fusion gene, but the behavior of the repeat upon replication might be influenced by additional flanking sequences. A way to circumvent these influences would be to generate a mouse model with an expanded CGG repeat in the endogenous *Fmr1* gene. However, this is for technical reasons extremely difficult. Homologous recombination by itself is not difficult [12], but it is hard to generate a large amount of a long CGG repeat by PCR; a repeat up to 120 units can be amplified with the technique used, but the product can only be visualized by radioactive PCR. To achieve homologous recombination, the repeat has to be cloned in a fragment of DNA containing the promoter and propagated in *E. coli*. The repeat in *E. coli* is very unstable, and it is almost impossible to isolate enough DNA with a long repeat necessary for homologous recombination.

The variation in the CGG repeat is polar, and it has been found that the repeat is unstable at the 3′ end [23]. This might be influenced by the direction of replication of the repeat sequence. The direction of replication has shown to be important for inheritance of CGG repeats in *E. coli*. When the CGG strand is in the leading strand, it tends to behave more stable. The repeat is highly instable, showing deletions and expansions when the CCG strand is in the leading strand [21, 33]. This might also play a role in mice and humans as well. Although we describe three independent transgenic strains, the stable inheritance can still be a consequence of the site of integration and the direction of replication in that region. It has been suggested that slippage events during replication are causing the instability of the CGG repeat. Misalignment of the repeat during replication might lead to continued polymerization or skipping of template sequences. If this is followed by an incorrect repair, repeat expansion can occur. Crossing of transgenic animals carrying CGG repeats and mice deficient in mismatch repair systems might reveal pathways that play an essential role in generating repeat instability.

In this study, we were not able to generate a mouse model to study the instable behavior of the CGG repeat present in the *FMR1* promoter. It is known that repeat expansions occur somewhere between gametogenesis and early embryogenesis. To study this, an animal model is required. Only then it will be possible to look at specific time points during development.

Furthermore, the differences in repeat stability upon paternal and maternal transmission and the importance of the methylation of the repeat remain to be elucidated. These differences can only be studied when a lot of material at specific points of development is available. The only way to obtain this material is the presence of an animal model which will enable us to study the behavior of the repeat and possibly to identify factors involved in the mechanism of CGG repeat expansion.

Acknowledgments

We like to thank Dr. Hergersberg for the generous gift of plasmid *FMR1/lacZ*, and C. Bakker and S. Mohkamsing for technical assistance. This study was supported by the Foundation of Clinical Genetics (E.d.G), by a BIOMED2 grant (B.A.O) and by the Netherlands Organization for Scientific Research (C.B.).

References

1 Turner G, Webb T, Wake S, Robinson H: Prevalence of fragile X syndrome. Am J Med Genet 1996;64:196–197.
2 Hagerman RJ: Physical and behavioral phenotype; in Hagerman RJ, Silverman AC (eds): Fragile X Syndrome: Diagnosis, Treatment and Research. Baltimore, Johns Hopkins University Press, 1996, pp 3–87.
3 Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang FP, Eussen BE, Van Ommen GJB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Galjaard H, Caskey CT, Nelson DL, Oostra BA, Warren ST: Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 1991;65:905–914.
4 Vincent A, Heitz D, Petit C, Kretz C, Oberlé I, Mandel JL: Abnormal pattern detected in fragile-X patients by pulsed-field gel electrophoresis. Nature 1991;349:624–626.
5 Hansen RS, Gartler SM, Scott CR, Chen SH, Laird CD: Methylation analysis of CGG sites in the CpG island of the human FMR1 gene. Hum Mol Genet 1992;1:571–578.
6 Sutcliffe JS, Nelson DL, Zhang F, Pieretti M, Caskey CT, Saxe D, Warren ST: DNA methylation represses FMR-1 transcription in fragile X syndrome. Hum Mol Genet 1992;1:397–400.

7 Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL: The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 1993;4:335–340.
8 Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, Boue J, Bertheas MF, Mandel JL: Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 1991;252:1097–1102.
9 Fu YH, Kuhl DP, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, Verkerk AJ, Holden JJ, Fenwick R Jr, Warren ST, Oostra BA, Nelson DL, Caskey CT: Variation of the CGG repeat at the fragile X site results in genetic instability: Resolution of the Sherman paradox. Cell 1991; 67:1047–1058.
10 Malter HE, Iber JC, Willemsen R, de Graaff E, Tarleton JC, Leisti J, Warren ST, Oostra BA: Characterization of the full fragile X syndrome mutation in fetal gametes. Nat Genet 1997;15: 165–169.
11 Reyniers E, Vits L, De Boulle K, Van Roy B, Van Velzen D, de Graaff E, Verkerk AJMH, Jorens HZ, Darby JK, Oostra BA, Willems PJ: The full mutation in the FMR-1 gene of male fragile X patients is absent in their sperm. Nat Genet 1993;4:143–146.
12 Bakker CE, Verheij C, Willemsen R, Vanderhelm R, Oerlemans F, Vermey M, Bygrave A, Hoogeveen AT, Oostra BA, Reyniers E, Deboulle K, Dhooge R, Cras P, Van Velzen D, Nagels G, Martin JJ, Dedeyn PP, Darby JK, Willems PJ: Fmr1 knockout mice: A model to study fragile X mental retardation. Cell 1994; 78:23–33.
13 Meijer H, De Graaff E, Merckx DML, Jongbloed RJE, De Die-Smulders CEM, Engelen JJM, Fryns JP, Curfs PMG, Oostra BA: A deletion of 1.6 kb proximal to the CGG repeat of the FMR1 gene causes the clinical phenotype of the fragile X syndrome. Hum Mol Genet 1994;3:615–620.
14 Rousseau F, Heitz D, Biancalana V, Blumenfeld S, Kretz C, Boue J, Tommerup N, Van Der Hagen C, DeLozier-Blanchet C, Croquette MF, Gilgenkranz S, Jalbert P, Voelckel MA, Oberlé I, Mandel JL: Direct diagnosis by DNA analysis of the fragile X syndrome of mental retardation. N Engl J Med 1991;325:1673–1681.
15 Nolin SL, Glicksman A, Houck GE, Brown WT, Dobkin CS: Mosaicism in fragile X affected males. Am J Med Genet 1994;51:509–512.
16 Wöhrle D, Hirst MC, Kennerknecht I, Davies KE, Steinbach P: Genotype mosaicism in fragile X fetal tissues. Hum Genet 1992;89:114–116.
17 Wöhrle D, Hennig I, Vogel W, Steinbach P: Mitotic stability of fragile X mutations in differentiated cells indicates early post-conceptional trinucleotide repeat expansion. Nat Genet 1993;4:140–142.
18 Wöhrle D, Schwemmle S, Steinbach P: DNA methylation and triplet repeat stability: New proposals addressing actual questions on the CGG repeat of fragile X syndrome (letter to the editor). Am J Med Genet 1996;64:266–267.
19 Hergersberg M, Matsuo K, Gassmann M, Schaffner W, Luscher B, Rulicke T, Aguzzi A: Tissue-specific expression of a FMR1/beta-galactosidase fusion gene in transgenic mice. Hum Mol Genet 1995;4:359–366.
20 Hornstra IK, Nelson DL, Warren ST, Yang TP: High resolution methylation analysis of the FMR1 gene trinucleotide repeat region in fragile X syndrome. Hum Mol Genet 1993;2: 1659–1665.
21 Shimizu M, Gellibolian R, Oostra BA, Wells RD: Cloning and characterization, and properties of plasmids containing CGG triplet repeats from the FMR-1 gene. J Mol Biol 1996;258: 614–626.
22 Deelen W, Bakker C, Halley D, Oostra BA: Conservation of CGG region in FMR1 gene in mammals. Am J Med Genet 1994;51:513–516.
23 Eichler EE, Hammond HA, Macpherson JN, Ward PA, Nelson DL: Population survey of the human FMR1 CGG repeat substructure suggests biased polarity for the loss of AGG interruption. Hum Mol Genet 1995;4:2199–2208.
24 Bingham PM, Scott MO, Wang S, McPhaul MJ, Wilson EM, Garbern JY, Merry DE, Fischbeck KH: Stability of an expanded trinucleotide repeat in the androgen receptor gene in transgenic mice. Nat Genet 1995;9:191–196.
25 Goldberg YP, Kalchman MA, Metzler M, Nasir J, Zeisler J, Graham R, Koide HB, O'Kusky J, Sharp CA, Jirik F, Hayden MR: Absence of disease phenotype and intergenerational stability of the CAG repeat in transgenic mice expressing the human Huntington disease transcript. Hum Mol Genet 1996;5:177–185.
26 Burright EN, Clark HB, Servadio A, Matilla T, Feddersen RM, Yunis WS, Duvick LA, Zoghbi HY, Orr HT: *SCA1* transgenic mice: A model for neurodegeneration caused by an expanded CAG trinucleotide repeat. Cell 1995;82:937–948.
27 Gourdon G, Radvanyi F, Lia AS, Duros C, Blanche M, Abitbol M, Junien C, Hofmann-Radvanyi H: Moderate intergenerational and somatic instability of a 55-CTG repeat in transgenic mice. Nat Genet 1997;15:190–192.
28 Monckton DG, Coolbaugh MI, Ashizawa KT, Siciliano MJ, Caskey CT: Hypermutable myotonic dystrophy CTG repeats in transgenic mice. Nat Genet 1997;15:193–196.
29 Mangiarini L, Sathasivam K, Mahal A, Mott R, Seller M, Bates GP: Instability of highly expanded CAG repeats in mice transgenic for the Huntington's disease mutation. Nat Genet 1997;15:197–200.
30 Zhong N, Yang WH, Dobkin C, Brown WT: Fragile X gene instability: Anchoring AGGs and linked microsatellites. Am J Hum Genet 1995;57:351–361.
31 Kunst CB, Warren ST: Cryptic and polar variation of the fragile X repeat could result in predisposing normal alleles. Cell 1994;77:853–861.
32 Eichler EE, Kunst CB, Lugenbeel KA, Ryder OA, Davison D, Warren ST, Nelson DL: Evolution of the cryptic FMR1 CGG repeat. Nat Genet 1995;11:301–308.
33 Wells RD: Molecular basis of genetic instability of triplet repeats. J Biol Chem 1996;271: 2875–2878.