Fully expanded FMR1 CGG repeats exhibit a length- and differentiation-dependent instability in cell hybrids that is independent of DNA methylation

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The fragile X syndrome is characterized at the molecular level by expansion and methylation of a CGG trinucleotide repeat located within the FMR1 locus. The tissues of most full mutation carriers are mosaic for repeat size, but these mutational patterns tend to be well conserved when comparing multiple tissues within an individual. Moreover, full mutation alleles are stable in cultured fibroblasts. These observations have been used to suggest that fragile X CGG repeat instability normally is limited to a period during early embryogenesis. DNA methylation of the repeat region is also believed to occur during early development, and some experimental evidence indicates that this modification may stabilize the repeats. To study the behavior of full mutation alleles in mitotic cells, we generated human–mouse somatic cell hybrids that carry both methylated and unmethylated full mutation FMR1 alleles. We observed considerable repeat instability and analyzed repeat dynamics in the hybrids as a function of DNA methylation, repeat length and cellular differentiation. Our results indicate that although DNA methylation does correlate with stability in primary human fibroblasts, it does not do so in the cell hybrids. Instead, repeat stability in the hybrids is dependent on repeat length, except in an undifferentiated cellular background where large alleles are maintained with a high degree of stability. This stability is lost when the cells undergo differentiation. These results indicate that the determinants of CGG repeat stability are more complex than generally believed, and suggest an unexpected role for cellular differentiation in this process.

INTRODUCTION

Expansions of trinucleotide repeat sequences are responsible for a growing list of human diseases including the fragile X syndrome, Huntington’s disease, myotonic dystrophy and a number of ataxias (1,2). The sequence of the repeats and the mechanisms by which the expansions cause disease vary among the disorders. Each of the repeats is polymorphic within normal populations, but when expanded beyond critical size thresholds have the ability to undergo large intergenerational changes in repeat number as well as somatic changes within an individual (1–3). Somatic instability is most pronounced in the fragile X syndrome and myotonic dystrophy, where it results in a high degree of mosaicism in most affected individuals such that the length of the repeat tract can vary widely among different cell populations within an individual.

The molecular basis of repeat instability is not well understood nor is it known whether somatic and intergenerational instability share in common any mechanistic similarities. Most information regarding the mechanisms of repeat dynamics has come from the study of cloned repeat tracts replicated in Escherichia coli or Saccharomyces cerevisiae. Repeat stability in these organisms is modulated by both cis- and trans-acting factors. Important cis parameters include the length and sequence composition of the repeat (4–7) as well as the direction of replication (4,6–9). Trans-acting factors include mutations in genes that encode DNA repair proteins (10–12), RAD27 (an enzyme involved in processing Okazaki fragments) (13,14) and the SbcC endonuclease (15). Several mouse models designed to study trinucleotide repeat dynamics in a mammalian context have also been reported. These models have been based on transgenic animals that carry trinucleotide repeat sequences, usually within the context of human genomic DNA or cDNA. The success of these models with respect to repeat instability has been mixed, with some CAG transgenics (16–19) and all CGG transgenics (20–22) showing no instability. Modest somatic and intergenerational changes in CAG and CTG repeat number have been reported in other transgenic models (23–29). However, the changes tend to be small and have not yet reproduced the large intergenerational expansions observed in humans.

The CGG trinucleotide repeat that causes the fragile X syndrome is located within the 5′-untranslated region of the FMR1 gene (30–33). In normal populations, the repeat tract is stable on transmission and is comprised of ~5–55 triplets (34). Pre-mutation alleles with ~55–220 repeats occur in unaffected carriers (34). When transmitted by a female, pre-mutation...
alleles can undergo large intergenerational changes in repeat number to form full mutation alleles with more than \(\sim 220\) repeats (31,34). Hypermethylation of full mutation alleles correlates with transcriptional silencing of the \(FMR1\) gene (35,36). The resulting reduction in or absence of the \(FMR1\) protein (FMRP) is believed sufficient to cause the fragile X syndrome phenotype (35,37,38). Somatic instability occurs in the majority of full mutation carriers, resulting in repeat length mosaicism, which is manifest as a complex smeared and multibanded hybridization pattern when examined by Southern blot analysis (34,39).

Numerous studies have found that the resulting hybridization patterns are well conserved among multiple tissues within individuals who carry typical methylated full mutations (39–47) and can be conserved in monozygous twins (41,48,49). A likely explanation for these observations is that the expanded repeats are maintained with little or no variability after an initial period of instability that presumably occurs during early embryogenesis. The analysis of cultured fibroblasts derived from fetal and adult full mutation carriers has demonstrated that methylated fragile \(X\) full mutation alleles are stable in these differentiated cells (43). Thus a demarcation appears to exist between embryonic cells in which somatic mosaicism is presumably produced by repeat instability and adult cells where the repeats are stable and the mosaic mutational patterns are maintained with little or no variation. Although the molecular basis of this boundary has not been determined, some experimental evidence suggests that DNA methylation stabilizes the repeats (50). Comparison of methylated and unmethylated alleles in heterogeneous cell populations has shown the mutational patterns to be more smeared and diffuse when the alleles are unmethylated (51,52). Moreover, post-mortem analysis of one fragile \(X\) male with a partially unmethylated expansion has identified inter-tissue differences in mutation patterns (53). If methylation influences repeat stability, somatic variation might normally occur during the period of global demethylation in early development. According to this hypothesis, de novo methylation of the repeats at about the time of blastocyst implantation would fix in place any size variability that had occurred (50). In the rare individuals that harbor an unmethylated expansion, the period of repeat instability would be extended and result in both inter-tissue heterogeneity and repeat instability in cultured cells.

We report the dynamic behavior of the fragile \(X\) CGG repeat in a tissue culture system. Repeat stability of methylated and unmethylated alleles was examined in both primary human fibroblasts and human–mouse cell hybrids. We show that methylation and repeat stability are correlated in the human cells, but in the cell hybrids both methylated and unmethylated alleles are highly unstable. Instability in the hybrids occurs by both expansion and contraction, and is a function of repeat length and cellular differentiation.

**RESULTS**

DNA methylation predicts repeat stability in primary human fibroblasts

To assess the proposed relationship between methylation and repeat stability in human cells, clonal fibroblast lines were isolated from primary skin cultures of three males bearing full mutations. Two of the males carry typical full mutations with complete methylation at the diagnostic \(EagI\) site (data not shown). The third individual (M.K.) is a high-functioning male who carries a full mutation that is unmethylated not only at the \(EagI\) site, but also throughout the CpG island and the CGG repeat (54). For each cell line, clonal isolates were generated by seeding the cells at low density and later harvesting individual clones with glass cylinders. The clonally derived cell cultures were expanded through \(\sim 24\) population doublings until confluent in a \(75\) cm\(^2\) flask and then were harvested for DNA isolation. Repeat stability in the clones was assessed by Southern blot analysis of genomic DNA digested with either \(PstI\) alone or with \(EcoRI\) and \(EagI\) (Fig. 1A). Analysis of DNA digested with \(EcoRI\)–\(EagI\) also allows an assessment of methylation status at the \(EagI\) site. Hybridization with probe \(pfxa3\) revealed discreet bands with no smearing in 14 clones isolated from the two methylated full mutation carriers. Seven of the clones are shown in Figure 1B. The sample in lane 7 contains two hybridization bands that most probably are the result of an impure clone rather than of repeat instability. These results are consistent with a previous report of repeat stability in cultured fibroblasts (43). The clonal cell DNA samples isolated from the hypomethylated full mutation male, M.K., showed band patterns with prominent smearing and in some cases samples with multiple allele sizes. The complexity of the patterns is consistent with repeat instability generating extensive repeat length mosaicism during clonal proliferation of the cultures. Thirty-seven M.K. fibroblast clones were analyzed, of which 26 contained smearing and/or multiple bands. Seven representative clones are shown in Figure 1C. Although it is formally possible that some of these cases of multiple alleles are due to impure clones, a comparison of the hybridization patterns of the methylated alleles (one of 14 with more than one hybridization band, no smearing) with the unmethylated alleles (26 of 37 with multiple bands and/or smearing) demonstrates that methylation status correlates with repeat stability in human fibroblasts.

Methylated repeats become unstable in differentiated cell hybrids as a function of size

The study of repeat dynamics in primary human fibroblasts is limited by their finite replicative capacity. To characterize better the repeat length changes, we sought a system in which repeat stability could be studied in different cellular backgrounds and over a large number of cell divisions. Recognizing that chromosomal context might have an influence on repeat behavior (3) and that it is not possible at this time to amplify and clone large CGG repeats (6,7), we immortalized human fragile \(X\) chromosomes by whole-cell fusion with a differentiated mouse cell line (Diff6). Retention of the human \(X\) chromosome in the hybrids was selected on the basis of the \(HPRT\) locus, which is \(\sim 19\) Mb centromeric of the \(FMR1\) gene.

Human–mouse hybrids were obtained in fusions with fibroblasts from two males bearing full mutations (TC38-89, GM04026) and a female bearing a full mutation and a normal allele (GM05847A). A total of 62 hybrid clones was obtained. Southern blot analysis with probe \(pfxa3\) demonstrated the presence of the human \(FMR1\) CGG repeat region in 60 of 62 hybrids, as well as a non-polymorphic mouse-specific band at 5.6 or 1.2 kb in \(PstI\)- or \(EcoRI\)–\(EagI\)-digested DNA, respectively.
All expanded fragile X alleles maintained complete methylation at the Eagl site. Despite methylation, hybridization patterns in 40 of the 60 hybrid clones were smeared (data not shown) and thus highly suggestive of instability. The chromosome content of two unstable hybrid clones was evaluated by fluorescence in situ hybridization (FISH). We examined 20 metaphases from each hybrid and found the mean number of human X chromosomes and autosomes to be 1 and 15, respectively. This result indicates that the smeared and poly-banded hybridization patterns that demonstrate instability are not explained by the presence of multiple human X chromosomes in the hybrids.

Because the hybrid clones have a much greater replicative capacity than primary human fibroblasts, repeat length mosaicism that occurs during clonal proliferation can be demonstrated more clearly by examining allele sizes in subclones. Although repeat instability continues during expansion of the subclones, a random assortment of subclones provides information about the magnitude and direction of repeat length change that has occurred after cell fusion. Repeat stability in nine hybrid clones with allele sizes ranging from 280 to 1610 repeats was examined further by subcloning. Southern blot analysis of parental and subclone DNA samples showed a large number of contractions and a few expansion products in many subclone lanes (Fig. 2). These changes were more frequent and occurred with greater magnitude when the parental hybrid clones began with large alleles (Table 1). The relationship between length of the parental repeat and the frequency (Fig. 3A) and magnitude (Fig. 3B) of change in the subclones is best described with a logarithmic function.

In some subclones, contraction events created small and apparently stabilized alleles with notably sharp and intense hybridization bands. The stability of three contraction products with 320, 270 and 170 repeats was examined by subcloning the cells a second time to create secondary subclones. In each case, the small alleles maintained complete methylation of the Eagl site and were quite stable, with no instability observed in all of 10 secondary subclones containing a 170 repeat pre-mutation sized allele (Fig. 2 and Table 1). These results demonstrate that a large unstable allele can be stabilized by contraction to a size below a stability threshold estimated to be ~200–250 CGG repeats (i.e. the size of a large pre-mutation).
Instability of large methylated repeat alleles is a function of cellular differentiation

To assess repeat behavior in an undifferentiated cellular background, chromosomes bearing methylated fragile X full mutation alleles were transferred to the undifferentiated DelTG3 embryonal carcinoma (EC) cell line by whole-cell fusion. Both Dif6 and DelTG3 are derivatives of the P19 EC cell line. Fusion to the EC cells was less efficient than to the differentiated cells and generated three hybrid clones. Two of the hybrids maintained the undifferentiated morphology characteristic of EC cells (Fig. 4A) and the third underwent spontaneous differentiation (Fig. 4D). Each hybrid clone was expanded through ~24 population doublings and harvested for DNA isolation and cryopreservation. Southern blot analysis showed the presence of large alleles (600–800 repeats) with complete methylation at the\textit{Eag}I site in each of the clones (data not shown). The chromosome content of one clone was studied by FISH analysis of 20 metaphase preparations. The mean number of human X chromosomes per cell was one and the mean number of human autosomes was 10.

Repeat stability in the hybrid clones was assessed with the subcloning strategy described previously. Subclones derived from the undifferentiated hybrid clones maintained the parental repeat size with little variation despite carrying full mutation alleles of sufficient size to be highly unstable in the Dif6 background (Fig. 4B and C; Table 1). In contrast to the repeat stability observed in the EC background, subclones derived from the spontaneously differentiated hybrid clone contained a highly unstable repeat (Fig. 4E). This result suggested that the human fragile X CGG repeat is maintained with differential stability in differentiated and undifferentiated hybrid cells with the same genetic background. The destabilizing effect of differentiation was confirmed by treating one of the undifferentiated hybrid clones (containing 740 repeats) with retinoic acid (RA) to induce differentiation. Differentiated subclones were isolated, and an\textit{Eco}RI–\textit{Eag}I Southern blot showed the presence of novel allele sizes in the subclones with an average magnitude of change of 230 repeats, consistent with significant repeat instability (Figs 3 and 4F; Table 1). In comparison, the two undifferentiated subclones that exhibited instability revealed contractions of only 40 repeats.

Unmethylated repeats are unstable in differentiated cell hybrids

Forty-seven hybrid clones were isolated from fusions between the Dif6 differentiated mouse cells and the M.K. fibroblasts containing unmethylated full mutation alleles. Southern blot analysis with probe \textit{pfxa3} demonstrated the presence of the human \textit{FMR1} CGG repeats in 45 of the 47 clones. Allele sizes were observed in the hybrids at frequencies proportional to their relative abundance in the primary fibroblast culture (Fig. 1C, lane 3). Thus most hybrid clones contained alleles with 300–350 repeats and a few contained large alleles of 600–750 repeats. Subclones were generated from four M.K. hybrid clones with repeat sizes of 740, 730, 350 and 330 triplets. The subclones were expanded through an additional 24 population doublings until DNA was isolated. Repeat stability in the subclones was assessed by Southern blot of \textit{Eco}RI–\textit{Eag}I-digested genomic DNA. Comparison of the allele size in the parental hybrid clone with those observed in the subclones
again revealed expansion and contraction products in the subclones that are not visible in the parental clone (Fig. 5). Like the methylated alleles in the differentiated cell hybrids, both expansion and contraction products were observed in the subclones and occur with greatest frequency and magnitude when derived from parental clones that contain larger alleles (Table 1).

**DISCUSSION**

The study of dynamic size changes in the *FMR1* CGG repeat tract has been complicated by several factors. Most notable among these is the inability to isolate large expansions with molecular approaches. We have bypassed this barrier by working with X chromosomes bearing *FMR1* expansions, and in doing so have made three novel and unexpected observations: (i) high levels of CGG repeat instability occur in human–mouse hybrid cells containing a majority of mouse chromosomes; (ii) this instability is independent of DNA methylation status; and (iii) cellular differentiation triggers repeat instability in the hybrid cells. These results are particularly surprising because work with primary human fibroblasts suggested that DNA methylation status was a prime determinant of repeat stability, i.e. methylated repeats are stable and unmethylated repeats are unstable in these primary cells (43,51,52). Others have used similar observations in cultured fibroblasts, and the observation that complex size patterns are conserved between many tissues within typical fragile X patients, to propose that repeat size instability occurs very early in development (41,43,55) and is restricted by DNA methylation (50). This methylation event is presumed to occur at about the time of blastocyst implantation when genome-wide methylation is known to occur (56). Prior to implantation, the genome is largely hypomethylated (56), and it is during this time that repeat instability is predicted to occur.

It is difficult to devise a simple model to reconcile the differences in behavior of the expanded repeats in the hybrid cells versus the fibroblasts. Although it is tempting simply to invoke the difference in genetic backgrounds, i.e. primary human fibroblasts versus immortal hybrid cells, the issue is clearly more complex because instability is only observed in the differentiated hybrids. Those hybrids that retained an embryonic cell morphology maintained the repeats with a high level of stability despite containing a genetic background identical to that of the differentiated mouse cells in which the repeats are unstable. Therefore, genetic background cannot provide the sole explanation for repeat instability in human–mouse hybrids. It is also not possible to use DNA methylation status to explain our results because methylated expansions are stable in the undifferentiated cells yet highly unstable in differentiated

### Table 1. Analysis of repeat length changes in fusion subclones

<table>
<thead>
<tr>
<th>Parental clone</th>
<th>Subclones</th>
<th>Parental clone length</th>
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<th>Mean magnitude of change</th>
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<td></td>
<td></td>
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<td>Larger</td>
<td>Smaller</td>
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cells. Moreover, methylated alleles shifted from stable to unstable upon cellular differentiation in vitro. In total, the results with the hybrid cells suggest that trans-acting, cell-specific factors provide a key role in controlling repeat stability instead of a cis-acting factor such as DNA methylation. If so, these factors could be missing from the differentiated mouse cells that were used as well as those rare human cells containing unmethylated full expansions. In this regard, it is important to note that DNA methylation is apparently normal outside of the region immediately surrounding the unmethylated expansion (54), suggesting again that this cis modification does not by itself control stability of the region. However, this does not rule out a role for trans-acting factors that recognize DNA methylation, such as methylation binding proteins (57).

Another potential, and perhaps related, set of trans-acting factors controlling repeat stability could be those involved in determining DNA replication direction. It is known that replication direction has substantial influence on smaller trinucleotide repeat regions propagated in E.coli (4,6,7) and S.cerevisiae (8,9). It is also known that Drosophila melanogaster embryonic cells initiate replication at closer intervals than adult cells (58). Therefore, it is possible that repeat stability in the cells we have examined is a direct result of cell-specific factors that influence replication direction through the expanded CGG repeat. A clear and testable prediction is that this direction will switch at the Eag1 site, in contrast to bona fide pre-mutation alleles that are usually unmethylated. The 170 repeat pre-mutation allele and a 270 repeat full mutation allele were the only two alleles that exhibited complete stability in subclones. Based on these observations and the logarithmic line of best fit calculated from the methylated alleles in the Dif6 hybrids, we estimate a threshold for stability at ~200–250 CGG repeats. Interestingly, this threshold corresponds to the boundary in human cells between somatically stable pre-mutation alleles and somatically unstable full mutation alleles. It is also consistent with reports describing transgenic mice carrying pre-mutation alleles in which the repeats are stable both somatically and intergenerationally (20–22). In contrast, somatic instability has been observed in transgenic mice carrying human trinucleotide repeats with as few as 55 CTG (23) and 90 CAG (28) repeats. These results suggest fundamental differences in size-dependent repeat stability for CGG and CAG/CTG trinucleotide repeats.

Most of the repeat length changes observed in the hybrid cells were the result of repeat contraction. Subclones that contained contraction products outnumbered those that contained expansion products by seven to one. A tendency for contraction events has also been observed when triplet repeats are replicated in E.coli (4,6,7,10) and S.cerevisiae (8,9), and in cell hybrids that contain an expanded myotonic dystrophy repeat (60). The bias towards contraction products suggests that the mechanism of repeat instability in the hybrids is probably not unequal homologous exchange because this model predicts expansion and contraction products to be generated with equal frequency (61). Polymerase slippage, perhaps

Figure 3. Effect of CGG repeat length on instability. These diagrams are based on data in Table 1. Closed squares, methylated alleles in Dif6; open squares, unmethylated alleles in Dif6; closed triangles, methylated alleles in DelTG3 cells that have remained undifferentiated; closed inverted triangles, methylated alleles in DelTG3 cells that have differentiated. (A) Percentage of subclones in which the parental repeat length has been maintained with no change. (B) Mean magnitude of change observed in the subclones. The mean magnitude of change is calculated using the absolute value of the differences between parental and subclone bands and thus treats expansion and contraction products equally. The mean magnitude of change is presented as a percentage of the repeat length in the parental clone. In both panels, a logarithmic line of best fit is shown that is calculated from the methylated alleles in Dif6.
accompanied by the formation of secondary structures (61,62), would be more consistent with these data.

The contraction bias we have observed in the hybrid cells is also consistent with observations that male primordial germ cells of fragile X patients initially contain full mutations that undergo repeat contraction to produce spermatozoa with pre-mutation alleles (55,63). Although it is not known whether the mechanisms underlying somatic and germline repeat length variability share in common any mechanistic similarities, the apparent contraction bias in male primordial germ cells may occur mitotically. If the pre-mutation to full mutation transition occurs pre-zygotically, as suggested by some experimental evidence (39,63), then somatic mosaicism might also be a product of mitotic repeat contraction. Under this model, the final repeat lengths observed in somatic cells and male germ cells might represent different manifestations of the same underlying process where contraction continues unabated to produce pre-mutations in the germline while the contraction process is interrupted in the somatic cells by a so far unidentified stabilizing influence. To the extent that repeat instability in the cell hybrids is a general model of repeat behavior in mitotically dividing mammalian cells, study of the cell hybrids may allow analysis of pathways relevant to instability in the male germline. Of course, the important repeat length changes that might occur in the female germline are less well understood and may occur by distinct mechanisms. Given the contraction bias and stability threshold observed in the cell hybrids, it is not immediately apparent whether analysis of the cell hybrids will yield information that addresses mechanisms of repeat expansion in the female germline.

In conclusion, we have demonstrated that it is possible to generate eukaryotic cells containing unstable fragile X full mutation alleles that undergo both repeat contraction and expansion. In this hybrid cell system, repeat stability is a function of cellular differentiation and repeat size. DNA methylation appears to have no impact on repeat stability in the hybrids as opposed to the correlation of DNA methylation and repeat stability observed in the primary human fibroblasts. These results suggest that the determinants of somatic repeat instability at the FMR1 CGG repeat are more complicated than generally believed. They also suggest a model to identify novel determinants.

MATERIALS AND METHODS

Cell lines and cell culture

Human fibroblast cell lines were purchased from the Coriell Human Genetic Mutant Cell Repository (Camden, NJ; GM04026, GM05847A, GM09497) or were established, with informed consent, from skin biopsies of adult fragile X full mutation carriers (TC38-89, M.K.) referred for clinical
fragile X testing to either the Oregon Health Sciences University DNA Diagnostic Laboratory or the Kaiser Permanente Cytogenetics Laboratory. All human cell cultures were maintained at 37°C/5% CO₂ in α-minimal essential media (α-MEM) supplemented with 20% fetal calf serum (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine and 16 µg/ml gentamicin sulfate. Clonal lines were obtained from the mass cultures by seeding the cells at a concentration of 40–100 cells per 150 mm dish. Individual clones were isolated with glass cloning cylinders ~2 weeks after seeding, then expanded until confluent in a 75 cm² flask. Subclones were generated from the parental hybrid clones by seeding the cells at a concentration of 100–200 cells per 150 mm dish. Individual subclones were isolated with glass cloning cylinders ~2 weeks after seeding, then expanded until confluent in a 75 cm² flask and harvested for DNA isolation.

The Dif6 cell line is a morphologically differentiated and 6-thioguanine-resistant clone isolated from the mouse P19-derived EC cell line H4D2 (64). DelTG3 is an undifferentiated and 6-thioguanine-resistant clone also isolated from H4D2 (65,66). Fusion experiments utilizing these cell lines were carried out with derivatives that had been transfected with the bacterial neomycin (neo) gene, which confers resistance to geneticin (G418). All mouse cell cultures were maintained at 37°C/5% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum and 5% serum plus (JRH Biosciences).

Cell fusions and differentiation

Whole-cell fusions were accomplished by mixing human fragile X fibroblasts with mouse cell lines Dif6 or DelTG3 at cell ratios of 1:3, 1:1 and 3:1 with a total of 3 × 10⁵ cells per 25 cm² flask. After mixing, the cells were plated and incubated for ~6 h, and then fused by adding 1 ml of 50% polyethylene glycol (PEG 1450; Sigma, St Louis, MO) solution in α-MEM. The cultures were rinsed three times with phosphate-buffered saline (PBS), incubated overnight in non-selective media and transferred the next day to 150 mm dishes at a concentration of 1–2 × 10⁵ cells/dish. Selection for hybrid clones was applied ~48 h post-fusion with 10 µg/ml hypoxanthine (Sigma), 10 µg/ml azaserine (Sigma) and 1 mg/ml geneticin (Gibco BRL, Rockville, MD). Individual hybrid clones were isolated 11–14 days after initiating selection and were then maintained in media supplemented with hypoxanthine and azaserine to retain the human X chromosome. Clones were expanded to a 75 cm² flask for DNA isolation and a 25 cm² flask for cryopreservation in DMEM with 10% dimethylsulfoxide (DMSO; Sigma). Subclones were generated from the parental hybrid clones by seeding the cells at a concentration of 100–200 cells per 150 mm dish. Individual subclones were isolated with glass cloning cylinders ~2 weeks after seeding, then expanded until confluent in a 75 cm² flask and harvested for DNA isolation.

An undifferentiated hybrid clone created with DelTG3 was induced to differentiate by treatment for 7 days in DMEM supplemented with 1.0 µM all-trans RA (Sigma). Differentiated subclones were isolated as described, with RA treatment continuing for the first 4 days after the cells were seeded at the cloning dilution.

DNA isolation and analysis

Genomic DNA was isolated using either a standard phenol–chloroform extraction or the Puregene DNA Isolation kit (Genta Systems, Minneapolis, MN). Aliquots (10 µg) were digested with restriction endonucleases PstI (Boehringer Mannheim, Indianapolis, IN) or EcoRI (Boehringer Mannheim) and EagI (New England Biolabs, Beverly, MA). Digested DNA was precipitated with sodium acetate and ethanol and then aliquots of 1.5–2.5 µg were separated by electrophoresis in 1% agarose/TAE and transferred to Bionoy nylon membrane (Gibco BRL) with 5x SSC. Hybridizations with a 32P-radiolabeled probe (Random Prime Labelling kit; Boehringer Mannheim) were carried out at 65°C in Church buffer with bovine serum albumin (BSA) (hybridization buffer II) (67) supplemented with 100 µg/ml heat-denatured herring sperm DNA. Probe pfxa3 (a kind gift from David L. Nelson, Baylor College of Medicine, Houston, TX), a 585 bp XhoI–PstI fragment of pE5.1 (32,34), was used to detect restriction fragments containing the CGG repeat. Membranes were washed twice in low stringency wash buffer II (67) at room temperature and then twice at 68.5°C in high stringency wash buffer II (67) diluted to 0.6x. Membranes were exposed sequentially to a Molecular Dynamics (Sunnyvale, CA) phosphorimaging screen and then X-ray film (X-OMAT; Kodak, Rochester, NY) at ~70°C. Molecular weight estimations were obtained digitally from film images with the DNA-VIEW program version 21 (Dr Charles H. Brenner, http://www.ccnet.com/~cbrenner/ ). The DNA Analysis Marker

Figure 5. Unmethylated full mutation alleles are unstable in differentiated cell hybrids. Each panel is a Southern blot of DNA digested with EcoRI–EagI, and hybridized with probe pfxa3. The number of CGG repeats that corresponds to each ladder band is shown on the far right. As is the case in Figure 2, DNA samples derived from the parental hybrid clones are loaded in the left-most lanes and are labeled with the number of human CGG repeats. Subclones derived from the parental clones are shown in the lanes to the right. Control samples and mouse-specific hybridization bands are not shown. All of the samples are unmethylated at the diagnostic EagI site.
System (Gibco BRL) was used as the molecular weight standard ('ladder') for all of the Southern blot experiments.

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