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Dynamic behavior of fragile X full mutations in cultured female fetal fibroblasts

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ABSTRACT

AIM: To assess mitotic stability of the fragile X full mutations and its relationship with DNA methylation. **METHODS**: The length change of the expanded CGG repeats was examined and correlated it with the methylation status in the DNA samples isolated from the fibroblasts derived from a fragile X female fetus and a fragile X male adult, respectively. **RESULTS**: A dramatic instability of the expanded CGG repeats in the female fetal fibroblasts was observed. Southern blot analysis revealed that the 6.9-kb major expanded band detected in passage 2 was completely replaced by a 7.7-kb band after passage 30. Fibroblast clones derived from the passage 3 displayed an unstable expansion of the CGG repeat during clonal proliferation, while methylation status of the CGG repeat region was maintained. In contrast, in fragile X male fibroblasts the expanded CGG repeats were stable during clonal proliferation. **CONCLUSION**: The mitotic instability of expanded CGG repeat is not always restricted in early development window as proposed previously and other elements rather than DNA methylation could affect the stability of the expanded CGG repeats in fragile X female fetal fibroblast cells.

INTRODUCTION

The fragile X syndrome (OMIM 309550) 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 the repeat size, but these mutational patterns tend to be well conserved when comparing multiple tissues within an individual and between homozygous twins with the fragile X full mutation[1,2]. Full mutation alleles are shown to be stable in cultured male fibroblasts[3]. These observations have been used to suggest that instability of the expanded CGG repeat is limited to a period during early embryogenesis. DNA methylation of the repeat region that is also believed to occur during early development is supposed to stabilize the expanded repeats[3]. On the other hand, the repeat dynamics in the cell hybrids is found to present as a function of the repeat length and cellular differentiation, suggesting trans-acting, cell specific factors play a key role in controlling repeat stability[4]. However, the molecular basis of unstable expansion of the trinucleotide repeat sequences is still not well understood.

An unusual dynamic behavior of the fragile X full mutation drew our attention when we were studying the nonrandom X inactivation and selection of fragile X

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full mutation in fragile X female fetal fibroblast cells[5]. The purpose of this study was to assess the mitotic stability of expanded CGG repeats in the fragile X female fetal fibroblasts and correlate it with DNA methylation. The observations were mainly derived from our last study[5].

MATERIALS AND METHODS

Cell culture and cloning Fibroblast cultures were established from lung tissue of a 20-week fragile X female fetus and skin fibroblasts of an adult fragile X male, respectively. Experiments were permitted by Nanjing Medical University. Cells were maintained in MEM medium with 15 % fetal calf serum in 75-cm^2 tissue culture flasks and were subcultured by trypsinization with a split ratio of 1:2 in every 3 d. The fetal fibroblasts were grown to 34 passages and the adult cells were grown to 22 passages. Clonal cultures were obtained by dilute seeding of 200 cells in 10-cm diameter Petri dishes. Non-contiguous cell clones were marked and isolated using siliconized glass-cloning cylinders about two weeks after seeding, then expanded until confluent in 75-cm^2 tissue culture flasks and harvested for DNA isolation.

DNA isolation and analysis DNA was extracted from fibroblasts using a standard salting out procedure[5]. DNA 10 μg was digested either with *Hin*d III and the methylation sensitive enzyme *Xma* III or with *Pst* I alone (Fig 1). The digested DNA was separated in 0.8 %-1.0 % agarose gels and transferred onto Hybond^{TM}-N$^+$membranes (Amersham) by alkali blotting. The transferred DNA was hybridized with ^{32}P-radiolabeled DNA probe Ox0.55[1] at 65 ºC in a solution containing 5×SSPE, 5×Denhardt's buffer, 0.5 % SDS, and 500 mg/L herring sperm DNA. The membranes were washed in 0.1×SSC, and 0.1 % SDS at 65 ºC and exposed to Kodak XR5 film at -55 ºC for 2–3 d. Lamda *Hin*d III digests as well as 5.2 and 2.8-kb fragments from control females were used as sizing standards.

RESULTS

Instability of full mutation alleles in fragile X female fibroblasts We carefully examined the change in repeat length and methylation status of the CGG repeat in DNA samples isolated from different passages of serially cultured fibroblasts of the fragile X female fetus. Genomic DNA was digested with *Hin*d III/*Xam* III and probed with ^{32}P-labeled Ox0.55 in Southern blot. As shown in Fig 1, passage 2 displayed a heterogeneous smear with a major expansion of 6.9-kb and a discrete 5.9-kb band. Additionally, 2.8-kb and 5.2-kb fragments corresponding to normal active and inactive X chromosomes were detected. With further passages the mutation pattern underwent dramatic change. Evidence for *in vitro* expansion was observed. The 5.9-kb band disappeared from passage 9 onwards and the 6.9-kb band was completely replaced by a 7.7-kb band after passage 30 (Fig 1), indicating a significant increase in the length of the CGG repeat (from 500–750 repeats). Notably, digestion with methylation sensitive enzyme *Xma* III did not reveal any change of methylation status in the unstable CGG repeats, suggesting the mitotic instability was independent of methylation of the expanded CGG repeat. As expected, the wild type *FMR1* allele on the normal active X chromosome did not change its size (Fig 1). The 2.8-kb band was maintained throughout the study, providing an internal control. Disappear-

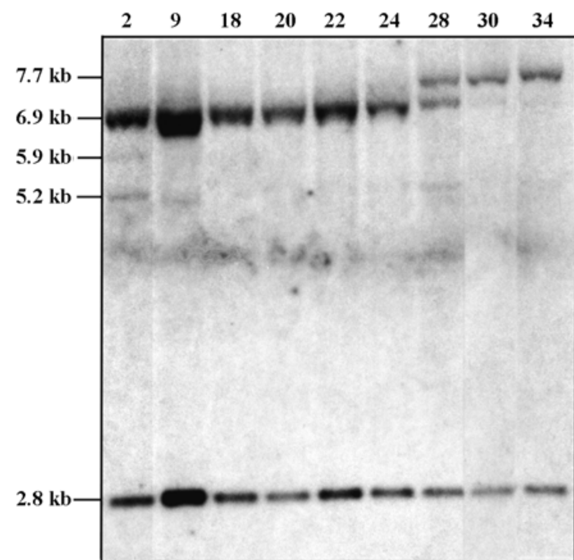

Fig 1. Mitotic instability of the fragile X alleles in serial cultivation of fibroblasts derived from a 20-week fragile X female fetus. DNA was digested with *Hind* III and methylation sensitive enzyme *Xam*III and probed with ^{32}p-labeled Ox0.55. The 2.8-kb and 5.2-kb bands represent the normal FMR-1 alleles on the normal active and inactive X-chromosomes, respectively. The passage number is indicated above the lanes. Passage 2 displayed a heterogeneous expansion pattern with a 6.9-kb major expanded band. With further passaging the expansion pattern changed and the 6.9-kb band was completely replaced by a 7.7-kb band after passage 30. The normal 2.8-kb band was unchanged throughout the study. So did the methylation status of the expanded FMR-1 alleles.

ance of the 5.2-kb band that represents the normal inactive X chromosome is known to result from selection in favor of cells with the normal active X chromosome[5].

As a mass culture usually contains multiple clones with different growth potential, we further analyzed the fibroblast clones derived from passage 3 of the same fragile X female cell line to confirm the dynamic expansion of CGG repeat observed in mass culture. The DNA isolated from 29 clones was digested with *Hin*d III/*Xma* III or *Pst* I alone and probed with Ox0.55. If the fragile X full mutation is stable in fibroblast cells, all individual clones should display a single sharp fragment instead of smear or multiple band patterns. However, continual expansion of the fragile X full mutations was again recognized in these clones. In Southern blotting of *Hin*d III/*Xma* III-digested DNA, 4 out of 29 clones display a smeared fragment with the size larger than that in the original culture, although most of the clones displayed a single fragment with a similar size to that in the original culture (Fig 2A, lanes 1, 2, 3, and 4). One of these clones presented two discrete expanded fragments (Fig 2A, lane 2). Southern blotting with *Pst* I-digested DNA, which is more sensitive than with *Hin*d *III*/*Xma* III-digested DNA for detecting the size variation of CGG repeats, further revealed the band patterns of smearing or multiple allele size in more than half of the examined clones (Fig 2B), indicating a mitotic instability that generated the repeat length mosaic during clonal proliferation. Our observation in fragile X female fetal fibroblast cells is in contrast to the earlier report on fragile X male fetal fibroblasts, where the full mutations were shown to be stable during the cell proliferation[3].

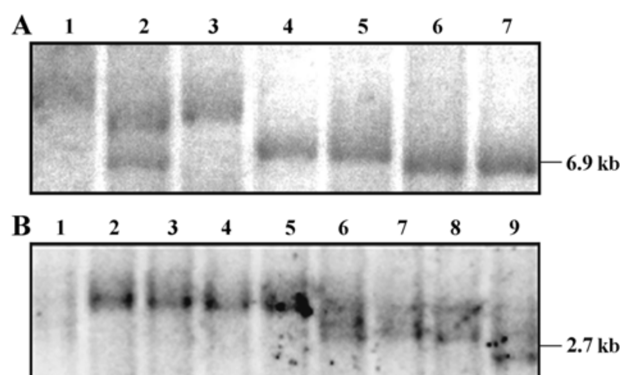

Fig 2. Mitotic instability of the fragile X alleles in fibroblast clones derived from the passage 3 of the same cell line described in Fig 1. The fragile X full mutation alleles are displayed only. (A) DNA was digested with *Hind* III and methylation sensitive enzyme *Xam* III and probed with ^{32}P-labeled Ox0.55. Four clones displayed blurred and smeared alleles that were larger than 6.9-kb in size (lanes 1, 2, 3 and 4) and one of them showed two discrete expanded alleles (lane 2), suggesting the full mutation alleles are unstable during clonal proliferation of the fragile X female fetal fibroblasts. The expanded alleles were methylated in all analyzed clones. (B) DNA was digested with *Pst* I and probed with ^{32}P-labeled Ox0.55. Smearing and multiple allele size was very obvious in the presented clones, confirming the observations described above further.

Stability of full mutation alleles in fragile X male fibroblasts To compare the mitotic stability of the expanded CGG repeats between male and female fragile X cells with the same experimental system we also studied the cultured fibroblasts derived from a fragile X male adult with the methods described above. DNA isolated from different passages of mass cultured fibroblasts was first analyzed by Southern blot. A 7.1-kb major expanded band with high methylation at *Xma* III site was constantly detected in different passages. The CGG repeat size was maintained from passage 3 to passage 22 (data not shown). Analysis of CGG repeat size in higher passages was limited by life span of the cell line established. The mitotic stability of the full mutation alleles was directly analyzed in fibroblast clones derived from passage 3 of the same fragile X male cell line. All 25 examined clones displayed single and sharp band in Southern blotting of *Pst* I-digested DNA (Fig 3). These data indicated the full mutation alleles were stable during clonal proliferation of the fragile X male fibroblasts and was consistent with the previous report [3].

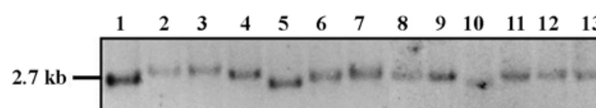

Fig 3. Southern blot analysis of the fragile X alleles in fibroblast clones derived from passage 3 of the fragile X male fibroblasts. DNA was digested with *Pst* I and probed by Ox0.55. All analyzed fibroblast clones displayed single and sharp band, indicating the full mutation alleles were stable during clonal proliferation of the fragile X male fibroblasts.

DISCUSSION

The expansions of trinucleotide repeats were known to be responsible for a growing list of human

diseases[6,7]. Understanding the molecular basis of CGG expansion would provide some important clues to reveal the mechanisms by which unstable expansion of the trinucleotide repeats caused human diseases. Our study revealed an important and interesting phenomenon regarding the mechanism underlying the unstable CGG expansion. We showed the experimental evidence that the fragile X full mutation experienced further expansion during the cell proliferation in cultured female fetal fibroblasts and this dynamic expansion was independent of DNA methylation. By contrast, the full mutations in the fragile X male fibroblasts were shown to be stable during the cell proliferation *in vitro* as previously reported[3].

Our observations had three implications: first, mitotic instability of the expanded CGG repeat was not always restricted in early developmental window as proposed previously, at least in female carriers of fragile X; second, other elements rather than DNA methylation could affect the behavior of the CGG expanded allele. Third, the intrinsic property of the cells, instead of the environmental effects *in vitro*, was responsible for the dynamic behavior of the CGG repeats observed in fragile X female fetal fibroblasts.

At present, it was still difficult to devise a simple explanation for our observation because of the unclear mechanism underlying the CGG expansion and the lack of required samples. It was possible that gene conversion played a role in the CGG repeat instability in the fragile X female fetal fibroblasts. Gene conversion in higher eukaryotes was referred to as a process of nonreciprocal transfer of genetic information. For genes showing extensive homology, one gene acts as a sequence donor, remaining unaffected, while the other gene received sequences and underwent variation[8].

Obviously, gene conversion required two basic conditions: genes showing extensive homology, and single or double strand breaks in the related chromosome. Amplified and methylated CGG repeats were associated with FRAXA and other four rare folate-sensitive fragile sites, which can cause instability *in vivo* and lead to chromosomal breakage[9]. Therefore, it was probable that gene conversion might occur between two X homologues in fragile X female cells and result in unstable expansion of the CGG repeats observed in this study. Actually, some studies implicated the role of gene conversion in mitotic instability of the trinucleotide repeats. For example, multiple exchanges between maternal X-chromosomes that very likely due to gene conversion, had been proved to cause a back mutation at the *FMR1* locus in fragile X female cells[8]. The risk of the unstable expansion of CAG repeats was increased by certain genotype, implying an inter-allelic interaction involved in the instability of the expanded CAG repeat[10].

In summary, our finding that a dynamic instability of the expanded CGG repeats occurred in the fragile X female fetal fibroblasts, but not in the fragile X male cells revealed an important phenomenon regarding the mechanism of unstable CGG expansion and suggested that gene conversion played a role in CGG expansion in fragile X female fibroblasts.

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