ATR protects the genome against CGG·CCG-repeat expansion in Fragile X premutation mice

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ABSTRACT

Fragile X mental retardation syndrome is a repeat expansion disease caused by expansion of a CGG·CCG-repeat tract in the 5′ UTR of the FMR1 gene. In humans, small expansions occur more frequently on paternal transmission while large expansions are exclusively maternal in origin. It has been suggested that expansion is the result of aberrant DNA replication, repair or recombination. To distinguish amongst these possibilities we crossed mice containing 120 CGG·CCG-repeats in the 5′ UTR of the mouse Fmr1 gene to mice with mutations in ATR, a protein important in the cellular response to stalled replication forks and bulky DNA lesions. We show here that ATR heterozygosity results in increased expansion rates of maternally, but not paternally, transmitted alleles. In addition, age-related somatic expansions occurred in mice of both genders that were not seen in ATR wild-type animals. Some ATR-sensitive expansion occurs in postmitotic cells including haploid gametes suggesting that aberrant DNA repair is responsible. Our data suggest that two mechanisms of repeat expansion exist that may explain the small and large expansions seen in humans. In addition, our data provide an explanation for the maternal bias of large expansions in humans and the lower incidence of these expansions in mice.

INTRODUCTION

Expansion of a tandem repeat array is responsible for disease pathology in the repeat expansion diseases, a group of genetic disorders that includes Fragile X mental retardation syndrome (FXS) (1,2). In FXS, the repeat unit is the triplet CGG·CCG. The mechanism responsible for expansion is unknown. However, it differs from the genome-wide microsatellite instability seen in diseases like hereditary non-polyposis colorectal carcinoma (HNPCC) in showing an expansion bias (more expansions than contractions) and occurring at a single genetic locus. In addition, at least in the case of mouse models for the disorders resulting from CAG·CTG-repeat expansion, mutations in DNA mismatch repair genes, like MSH2, MSH3 and PMS2, actually decrease the frequency of repeat expansion while the opposite is true for HNPCC (3–9).

Different diseases in this group involve repeats with different sequences and repeat unit sizes (10). These repeats have the potential to form secondary structures that are thought to play a role in the expansion process [see (11) for a recent review]. However, since not all of the repeats have the same properties, it is unclear whether all repeats expand via the same mechanism. Studies in bacteria and yeast have shown that variety of mechanisms can cause repeat instability in these organisms including DNA slippage during replication, errors in Okazaki fragment processing as well as aberrant DNA repair or recombination (12–42).

However, much is currently unknown about the events responsible for expansion in humans. For example, some expansion diseases only show small changes in repeat number on intergenerational transfer, while others result in alleles many times larger than the parental allele from which they are derived. The small expansions are typically seen when the repeats fall within an open reading frame, as in the case of the CAG·CTG-repeat responsible for Huntington disease (HD). These expansions show a paternal transmission bias. Large expansions, like those that cause FXS, are characteristically seen in regions outside of the open reading frame and occur almost exclusively on maternal transmission. Whether large and small expansions share the same mechanism is not known.

In addition, some diseases involve significant somatic instability while others do not and it is not known if the same mechanism is responsible for both germline and somatic expansion. The timing of intergenerational

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expansion is also unclear. Most studies have focussed on CAG·CTG-repeats. Some studies in transgenic mice have suggested that small expansions occur premeiotically in spermatogonia (9), whilst others suggest that expansions occur in haploid gametes (8,43). Some studies also suggest a second event occurs in the early female embryo. In some transgenic mouse models this event is an expansion (44) and in others it is a contraction (45,46). The differences in these two models has been attributed to the different genomic context of the repeats (44). This would be consistent with work in bacteria, yeast and tissue culture models that have implicated orientation, proximity to origins of replication and transcription as cis-acting factors affecting expansion (15,21,24,27,31,36,38,39,47,48).

In order to study the Fragile X repeat in as close to normal a chromosomal context as possible, we generated a FXS premutation knock-in (KI) mouse containing 120 CGG·CCG-repeats in the murine *Fmr1* gene (49). Since the murine *Fmr1* gene is located in a region of the X chromosome that is syntenic with the corresponding region of the human X chromosome, differences in *cis*-acting signals involved in expansion may be small. As was seen in other transgenic and Knockin mouse models of CGG·CCG-repeat expansion (50–53), instability in these animals resembles what is seen in humans in that the frequency is high and shows an expansion bias (49). In our mouse model large expansions that generate alleles in the full mutation range (>200 repeats) were seen, but at a much lower frequency than in humans (49). In fact most expansions in these mice are small, involving fewer than five repeats per generation and occurring more commonly in males than in females. In this respect these expansions resemble what is seen in human carriers of *FMR1* common or intermediate sized alleles (grey-zone alleles) (54) or those diseases such as HD that involve relatively small increases in repeat number.

Expansion occurring during the perigametic interval—between the last premeiotic mitosis and the first post-meiotic one—could explain the differences between mice and humans and the maternal CGG·CCG-repeat expansion bias in the human *FMR1* gene. This interval can last decades in human females creating a large window of opportunity for expansion to occur. In contrast, this interval lasts only months in female mice and in weeks in males of either species. Depletion of the DNA repair capacity for much of spermatogenesis could also exacerbate the differences in maternal and paternal expansion rates (55). It may also be that some of the differences between humans and mice reflect differences in the efficacy of DNA damage repair or checkpoint proteins.

One potential DNA damage checkpoint protein that may affect expansion frequency is the Ataxia-telangiectasia and rad3-related (ATR) kinase. ATR primarily responds to stalled replication forks and bulky DNA adducts like those arising from UV irradiation (56) or S(N)1-type alkylating agents (57). Here we show that *ATR* heterozygosity leads to increased maternally transmitted expansions and somatic expansions in mice of both genders that appear to involve aberrant DNA repair.

MATERIAL AND METHODS

Mice breeding and maintenance

The generation of the Fragile X premutation mice was described previously (49). The $ATR^{+/-}$ mice were a kind gift of Dr Eric Brown (Caltech, Pasadena, CA). Mice were maintained in accordance with the guidelines of the NIDDK Animal Care and Use Committee and with the *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, revised 1996).

Data analysis

Genomic DNA is prepared from mouse tail DNA or homogenized mouse tissue as previously described (49). Genotyping to detect the presence or absence of the disrupted *ATR* gene was carried out as described elsewhere (58). The primer pair, frax-c and frax-f (1), was used to detect both wildtype (WT) *Fmr1* and FXS premutation alleles. The size of the CGG·CCG-repeat tract was monitored by Polymerase chain reaction (PCR) using the primers frax-m4 (5′-CTTGAGGCCCAGCCGCCGT CGGCC-3′) and frax-m5 (5′-CGGGGGGGCGTGCGGTA ACGGCCCAA-3′). The binding sites for these primers are located immediately adjacent to the repeat tract and their 3′ ends are unique to the KI allele. The PCR reaction was done in one of two ways. Radiolabelled PCR products were generated by inclusion of α-P^{32}-dCTP in the reaction mix as previously described (49). The resultant PCR products were analysed on a 5% poly-acrylamide sequencing gels. PCR reactions were also carried out using one 6-carboxyfluorescein (FAM)-labelled primer. The reaction products were then run on a 3130XL Genetic Analyzer and analysed using GeneMapper® 3.7 (Applied Biosystems, Foster City, CA). Instability seen using the Genetic Analyzer was also clearly visible by conventional gel electrophoresis. Results were confirmed where necessary by Southern blotting. PCR across long repeats typically produces multiple bands. In young mice these bands show a Gaussian distribution about the mean that is very similar for alleles in the same size range. The mean size of each allele was calculated based on the mobility of the central band in the cluster. Comparison of the parental mean and the offspring's mean determined from samples run on the same gel, allows the changes in the offspring's allele to be reproducibly determined. Statistical analysis of instability was carried out using the Chi square and Student's *t*-tests.

RESULTS

The checkpoint protein ATR is responsible for activating pathways that lead to the repair of stalled DNA replication forks and bulky lesions in DNA. Thus the effect of *ATR* mutations on the expansions seen in Fragile X premutation mice may shed light on the mechanism of repeat expansion. *ATR* null mice die during early embryogenesis (58). However, since *ATR* heterozygous mice show a small increase in tumour incidence and a small decrease in overall survival it is apparent that some effects of ATR deficiency can be seen even in the

Table 1. Expansions of a premutation allele in WT and $ATR^{+/-}$ mice on paternal and maternal transmission

		Offspring					
Cross		**% mice with expansions**					**Mean no. repeats added**
Male × Female		**(1) Total**	**(2) Males**	**(3) Females**	**(4) $ATR^{+/-}$**	**(5) WT**	
1	*$Fmr1^w$, $ATR^{+/+}$ × $Fmr1^{w/p}$, $ATR^{+/+}$	$37^≠$	$39^‡$	$33^¶$	–	$37^Ø$	$2.2^•$
2	$Fmr1^w$, $ATR^{+/+}$ × $Fmr1^{w/p}$, $ATR^{+/-}$	$86^{≠,□}$	$89^‡$	$83^¶$	$96^\#$	$63^{Ø,\#}$	$5.0^•$
3	*$Fmr1^p$, $ATR^{+/+}$ × $Fmr1^{w/w}$, $ATR^{+/+}$	61	–	61	–	61	$3.1^§$
4	$Fmr1^p$, $ATR^{+/-}$ × $Fmr1^{w/w}$, $ATR^{+/+}$	$68^□$	–	68	69	67	$5.2^§$
5	$Fmr1^p$, $ATR^{+/+}$ × $Fmr1^{w/w}$, $ATR^{+/-}$	63	–	63	63	63	2.2

W: wildtype *Fmr1* allele; P: *Fmr1* premutation allele; -: indicates offspring from crosses that should not have either the premutation or a mutated ATR allele; *: Data source: Entezam *et al*. (47); $^{≠,□,‡,¶,Ø}$Numbers sharing one of these symbols were compared using the Chi-squared test and shown to be significantly different with a *P*-value <0.005. $^{•,§}$Numbers sharing these symbols were compared using the Student's *t*-test and shown to be significantly different with a *P*-value <0.005.

heterozygous state (58). We thus analysed the transmission of a FXS premutation allele with 120 CGG·CCG-repeats in mice heterozygous for a disrupted *ATR* gene. The results obtained for the repeat length changes in the offspring of these mice are summarized in Table 1.

Intergenerational instability

ATR heterozygosity had no effect on the deletion frequency and no effect of *ATR* heterozygosity was seen on the stability of the normal mouse *Fmr1* repeat (data not shown). In contrast, a significant increase in the expansion frequency was seen when the premutation allele was maternally transmitted in $ATR^{+/-}$ mice compared to WT mice (86% versus 37%; cross 2 versus cross 1 in Table 1). This suggests that ATR is normally involved in protecting the genome against intergenerational expansions in female mice carrying FXS premutation alleles. In contrast, the expansion frequency on paternal transmission by $ATR^{+/-}$ mice was not statistically different from mice WT with respect to *ATR* (68% versus 61%, cross 4 versus cross 3 in Table 1). There was an apparent increase in the average number of repeats added per expansion on both paternal and maternal transmission in *ATR* heterozygotes despite the fact that no increase in expansion frequency was seen on paternal transfer.

There was no significant gender bias in the expansion frequency in the offspring of $ATR^{+/-}$ mothers. This is similar to what is seen in with Fragile X repeats in humans (59) and differs from the male expansion bias seen in a transgenic mouse model of CAG·CTG-expansions (45). *ATR* WT males showed no increase in the transmission of an expanded CGG·CCG-allele when crossed to $ATR^{+/-}$ females (Cross 5 in Table 1).

An expansion frequency of 63% was seen in the WT progeny of females carrying the premutation who are heterozygous for *ATR* (Cross 2). This is significantly higher than the 37% seen in offspring of females WT for *ATR*. The expansion frequency was even higher in the $ATR^{+/-}$ offspring of $ATR^{+/-}$ mothers (96%, cross 2 in Table 1).

Somatic instability

To study the role of ATR in somatic instability we examined the size of the repeat in different organs of young (10 weeks old) and old (18 months old) $ATR^{+/-}$ mice. As can be seen from Figure 1A, very limited somatic instability was seen in the liver of young mice as evidenced by the very slightly skewed distribution of repeat sizes. No instability was detected in other tissues. Small pool PCR was also negative for instability (data not shown). Old mice showed much more significant changes in organs like brain, testes and liver. In some instances little remained of the original allele size (see brain sample in Figure 1C). These changes showed a clear expansion bias. We have previously shown that no such somatic instability was seen in mice of a similar age that were WT for *ATR* (49). In some organs, like the male brain, the expansions resulted in a shift of the average allele size without changing the basic monophasic distribution of allele sizes (Figure 1C). In organs like liver and testes a biphasic distribution of allele sizes was apparent (e.g. Figure 1C). Whether the biphasic distribution reflects a predisposition of certain cells within the organ to expand is currently under investigation. A biphasic distribution of somatic expansion products has been reported in the liver in a mouse model knockin mouse model for myotonic dystrophy, a CAG·CTG-expansion disorder, where it was attributed to changes in ploidy in a subset of liver cells (60).

DISCUSSION

We have shown that a maternal ATR insufficiency leads to an increase in the frequency of intergenerational expansions. *ATR* heterozygosity also causes the appearance of age-related expansion products in certain organs of older males and females.

A number of lines of evidence support a prezygotic origin for the ATR-sensitive intergenerational expansions seen in females. The elevated expansion frequency in *ATR* WT offspring of *ATR* heterozygous mothers (Cross 2, column 5 in Table 1 compared to Cross 1, column 5) demonstrates that expansion can occur prior to fertilization of the oocyte. This is supported by the fact that the paternal expansion frequency in mice WT for *ATR* is the same whether the dam is $ATR^{+/-}$ or WT for *ATR* (Cross 5 in Table 1).
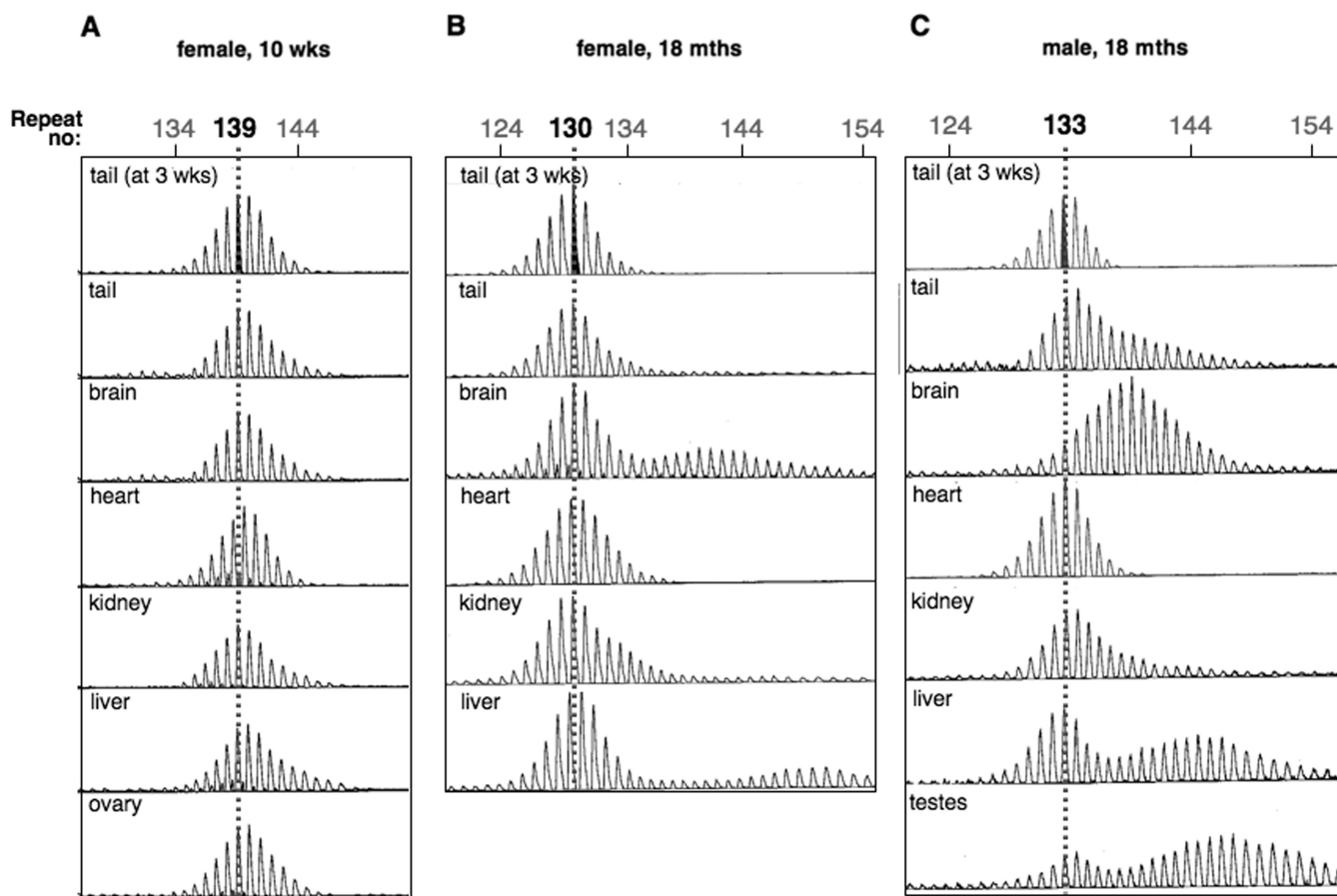
Figure 1. Somatic instability in $ATR^{+/-}$ mice carrying an FXS premutation allele. Genomic DNA was isolated from various organs of young (10 weeks old) and old (18 months old) FXS premutation mice and the repeat tract analysed by PCR using one FAM-labelled primer and an ABI GeneAnalyzer as described in the Materials and Methods. The number repeats in the modal allele in the tail DNA at 3 weeks of age is shown in black font and is indicated in the other organ samples by the grey dotted line. Similar results were obtained using PCR using ^{32}P-α-dCTP and denaturing gel electrophoresis as described previously (49).

Despite the maternal expansion bias, there is no gender bias in the likelihood of inheriting an expansion. This together with the fact that female mice do not show more somatic instability in adult tissues than males argues against somatic expansions that occur specifically either in the early female embryo or in the female germline prior to meiosis.

We have previously shown that male mice show no bias against the transmission of large repeats (49). Thus the female expansion bias in an $ATR^{+/-}$ background is not likely to be due to somatic expansions that are selected against in males. Furthermore, the excess of expansions in $ATR^{+/-}$ offspring (96% compared to 63% in ATR WT offspring) is not consistent with a somatic origin in the mother either since all her somatic cells would be $ATR^{+/-}$. Thus the most likely origin of the additional expansions in the $ATR^{+/-}$ offspring is in the haploid oocyte. Given that scheduled DNA replication does not occur in haploid gametes, DNA damage is thus likely to be responsible. The haploid gamete that gives rise to ATR WT offspring would not be ATR deficient. Thus if expansion were confined to this stage, we would not expect the expansion frequency to be higher than that seen in offspring of a mother WT for ATR. Therefore, some ATR-sensitive expansions in the WT offspring of $ATR^{+/-}$ mothers could have occurred in the oogonia or diploid oocyte. Female gametes remain diploid until just prior to ovulation. Thus the window of opportunity for expansions in these cells is much larger than it is for haploid gametes although the rate of expansion may in fact be lower.

It may be that the ATR heterozygosity in these mice reveals the existence of two different intergenerational expansion mechanisms, the first showing a higher expansion frequency in males that is less sensitive to ATR haploinsufficiency and the second, occurring predominantly in females, that is sensitive. This is not to say that ATR mutations are necessary for maternal expansions in humans, but rather that an ATR insufficiency in mice allows events that would have years to accumulate in humans, to be visible within the rodent lifespan.

The relatively ATR-insensitive mechanism may account for the paternal transmission bias seen with intermediate and grey-zone $FMR1$ alleles in humans (54). A higher mutation frequency in males is usually attributed to errors occurring during DNA replication since mature sperm are the product of more rounds of cell division than ova (61).

While it is possible that the increase in the average expansion size seen on paternal transmission reflects measurement errors, it may be that in males the ATR-deficiency simply delays the resolution of the replication problem. For example, in a strand-slippage scenario, such a delay could result in the incorporation of additional bases into the expanded allele without affecting the frequency with the initiation of strand-slippage occurs.

ATR-sensitive expansions may be more common in female mice since our data suggest that the mechanism responsible is related to the repair of DNA damage and not genomic replication. Expansions can thus occur at any point during gametogenesis, a process that lasts significantly longer in females. It is appealing to think of this mechanism as the basis for the strong maternal bias in the transmission of Fragile X full mutation alleles in humans. Since gametogenesis takes so much longer in human females, this may provide a much larger window of opportunity for such expansions to occur even in the presence of normal amounts of ATR.

Somatic expansion is also sensitive to *ATR* heterozygosity. One organ that showed evidence of significant somatic expansion is the brain. In some cases very little of the original allele was seen (see adult male brain in Figure 1C). Since a significant fraction of cells in the adult brain are post-mitotic, somatic expansion is also probably not limited to dividing cells. Thus these expansions may also arise from an aberrant DNA repair process rather than a problem with scheduled DNA replication. Expansion limited to some organs could be explained by differences in either the frequency with which the DNA damage that initiates expansion occurs in these organs or the frequency with which such mutations are repaired or eliminated. Organs such as liver and brain may be predisposed to expansion since *ATR* is expressed at a lower level in cells with a low proliferative capacity (62) and shows a lower affinity for chromatin in such cells (63).

In a transgenic mouse model of CAG·CTG-repeat expansion a deficiency of OGG1, an enzyme involved in the repair of the oxidation product of guanine, 7,8-dihydro-8-oxoguanine, reduces somatic expansion frequency (64). Oxidative DNA damage-induced expansion is likely to be high in organs like brain that in the Fragile X premutation mice show elevated levels of ATR-sensitive somatic mutations. However, since an OGG1 deficiency did not affect germline instability in the CAG–CAG-mouse model, its significance for intergenerational instability in the CAG·CTG-expansion diseases and in the etiology of Fragile X syndrome is unclear. In Fragile X premutation mice, an ATR insufficiency may prevent error-free DNA repair pathways from being activated to repair DNA damage, forcing the cell to use a secondary repair pathway that results in expansions. Potential pathways could be non-homologous end-joining or some sort of homologous recombination.

The effect of *ATR* mutations on the CGG·CCG-expansion frequency in female mice raises the possibility that DNA damage checkpoint proteins or proteins involved in DNA repair have the potential to affect expansion risk in humans. Such transacting factors may explain why the risk of expansion in premutation carriers is lower in those carriers identified by general prenatal screening than in carriers from known fragile X families (65). It could also explain why some intergenerational instability is apparent in some families with alleles in the 'grey zone' and not others (66) and the transition from an allele in the normal size range to a full mutation in two generations reported in one family (67). Our data also raise the possibility that there may be tissue variation in repeat lengths in older human premutation carriers that may be relevant for diagnosis and the severity of disease symptoms.

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