

ATM and ATR protect the genome against two different types of tandem repeat instability in Fragile X premutation mice

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ABSTRACT

Expansion of a tandem repeat tract is responsible for the Repeat Expansion diseases, a group of more than 20 human genetic disorders that includes those like Fragile X (FX) syndrome that result from repeat expansion in the *FMR1* gene. We have previously shown that the ATM and Rad3-related (ATR) checkpoint kinase protects the genome against one type of repeat expansion in a FX premutation mouse model. By crossing the FX premutation mice to Ataxia Telangiectasia-Mutated (*Atm*) mutant mice, we show here that ATM also prevents repeat expansion. However, our data suggest that the ATM-sensitive mechanism is different from the ATR-sensitive one. Specifically, the effect of the ATM deficiency is more marked when the premutation allele is paternally transmitted and expansions occur more frequently in male offspring regardless of the *Atm* genotype of the offspring. The gender effect is most consistent with a repair event occurring in the early embryo that is more efficient in females, perhaps as a result of the action of an X-linked DNA repair gene. Our data thus support the hypothesis that two different mechanisms of FX repeat expansion exist, an ATR-sensitive mechanism seen on maternal transmission and an ATM-sensitive mechanism that shows a male expansion bias.

INTRODUCTION

Tandem repeat expansion is the cause of 20 or more human genetic disorders, known collectively as the Repeat Expansion Diseases (1,2). This group of diseases includes the *FMR1* disorders, Fragile X (FX)-associated tremor and ataxia syndrome (FXTAS), FX associated

primary ovarian insufficiency (FXPOI) and Fragile X syndrome (FXS). Tandem repeat instability has also been implicated in a variety of other diseases including coronary artery disease, polycystic ovary disease, ADHD, type II diabetes and obesity (3–7). It is also a common source of human genetic variation that may affect gene expression but is not obviously pathological (8,9).

In the *FMR1* disorders, the affected repeat, which consists of the sequence CGG•CCG, is located in the 5' untranslated region of the X-linked *FMR1* gene. FXTAS and FXPOI occur when *FMR1* alleles have 55–200 repeats. Such alleles are referred to as premutation alleles. FXS, on the other hand, is seen when the repeat number exceeds 200 resulting in the generation of so-called full mutation alleles. There is a direct relationship between repeat number and the age of onset of FXTAS symptoms (10). There is also a relationship between repeat number and the age of menopause in FX premutation carriers, although this relationship ceases to be linear in the higher size ranges (11). There is also a direct relationship between the parental repeat number and the likelihood of expansion, with paternal transmission resulting in a higher frequency of small expansions (12), and large expansions occurring exclusively on maternal transmission. Deletions also occur, although the frequency is generally lower than that of expansions.

Many of the expansion-prone repeats form secondary structures including hairpins, tetraplexes and triplexes while less unstable repeats do not do so (13). This has led to the idea that secondary structures are somehow involved in repeat expansion. This would be consistent with the fact that many other unstable sequences also form similar structures (14,15). Many of the disease-associated repeats block DNA synthesis (16–19) and inhibit enzymes like the flap endonuclease FEN1 (20,21). Consistent with these findings, data from bacterial and yeast mutants suggests that problems with DNA replication, repair and recombination of these structure-forming sequences are all possible sources of repeat instability (22).

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However, the precise mechanism(s) responsible for repeat instability in mammals has not yet been identified.

We have previously shown that two different repeat expansion pathways exist in a knockin mouse model of the FX premutation (PM), one that occurs in both males and females, but that has a paternal transmission bias and the other that occurs exclusively in females (23). Evidence suggests that deletions involve a mechanism distinct from expansions (23,24). We also showed that the ATM and Rad3-related (ATR) DNA damage response protein is involved in reducing the incidence of maternal-specific expansions. ATR responds primarily to stalled replication forks and bulky DNA adducts that block DNA synthesis. A related protein, Ataxia Telangiectasia-Mutated (ATM) is involved in the response to double-strand breaks, although there is some functional overlap between the targets of the two proteins (25). In particular, ATM has been shown to be involved in the rescue of replication forks stalled by depletion of intracellular dCTP pools (26).

Here we show that ATM is also involved in reducing repeat expansions in mice. However, the ATM-sensitive expansions have characteristics quite different from the ATR-sensitive ones. This includes a gender difference in the expansion frequency that may be due to differences in the expression of an X-linked DNA repair gene. Our findings may have implications for the mechanisms of repeat expansion responsible for a number of genetic disorders in humans and for an important fraction of the genetic variation seen in human genomes.

MATERIALS AND METHODS

Mice breeding and maintenance

The generation of the FX premutation mice was described previously (24). The nomenclature *Fmr1*^{PM} is used to indicate the presence of the premutation allele. The *Atm*^{+/-} mice were obtained from Dr Andre Nussenzweig (NIH) from a colony originally obtained from Dr Anthony Wynshaw-Boris (27). 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). At least three different breeding pairs of each genotype were analyzed.

Data analysis

Genomic DNA is prepared from mouse tail DNA or homogenized mouse tissue as previously described (24). *Atm* genotyping was carried out as described elsewhere (27). The primer pair, frax-c and frax-f (28), was used to detect both wildtype (WT) *Fmr1* (*Fmr1*^{WT}) and FX premutation alleles (PM; *Fmr1*^{PM}). The size of the CGG•CCG-repeat tract was monitored by Polymerase chain reaction (PCR) using frax-m5 (5'-CGGGGGGCG TGCGGTAACGGCCCAA-3') and 6-carboxyfluorescein (FAM) or 4,7,2',4',5',7'-hexachloro-6-carboxyfluorescein (HEX) labeled frax-m4 (5'-CTTGAGGCCAGCCGCC GTCGGCC-3'). The binding sites for these primers are located immediately adjacent to the repeat tract and their 3' ends are unique to the PM allele. The reaction

products were then run on a 3130XL Genetic Analyzer and analyzed using GeneMapper® 3.7 (Applied Biosystems, Foster City, CA). Results were confirmed where necessary by Southern blotting. PCR across long repeats typically produces multiple bands and the size of each allele was calculated based on the mobility of the central band in the cluster. Statistical analysis was carried out using GraphPad QuickCalcs Software. A total of 91 and 85 paternal and maternal transmissions of the FX premutation allele from *Atm*^{+/+} parents and 71 paternal and 119 maternal transmissions from *Atm*^{+/-} parents were examined. The expansion and deletion frequencies were scored as the number of transmitted alleles that had undergone expansion or deletion divided by the total number of alleles scored. The number of error-free transmissions shown in Figure 1C, was scored as the number of alleles transmitted with no change in repeat number as a fraction of the total number of alleles transmitted.

RESULTS

In order to study the effect of ATM on the instability of FX premutation alleles, we have crossed mice containing ~130 CGG•CCG-repeats in the 5' UTR of the *Fmr1* gene (a *Fmr1*^{PM} allele) on the X chromosome (24) with mice carrying an *Atm* null mutation on chromosome 9 (27). Since *Atm* homozygous mutant mice are sterile (27), we were limited to studying intergenerational instability in animals that were heterozygous for *Atm*. Males and females carrying one *Fmr1*^{PM} allele and a disrupted *Atm* allele (*Atm*^{+/-}, *Fmr1*^{PM} males or *Atm*^{+/-}, *Fmr1*^{WT,PM} females) were then crossed to mice that were WT for both *Fmr1* and *Atm* (*Atm*^{+/+}, *Fmr1*^{WT} males or *Atm*^{+/+}, *Fmr1*^{WT,WT} females) as illustrated in Figure 1A. The repeat size in the progeny of these animals was then determined as previously described (23). These data were then used to derive the expansion frequency and mean number of repeats added to each expanded allele.

The expansion frequency seen on transmission of the FX premutation allele from an *Atm*^{+/-} male was significantly higher than that seen on paternal transmission from either *Atm*^{+/+} or *Atm*^{+/-} males [86% compared with 62 and 68%, respectively; *P* = 0.0036; Figure 1 and (23)]. Thus, despite the presence of one functional copy of the *Atm* gene, the number of expansions in the progeny of *Atm*^{+/-} males increased by 40%. Since the expansions and deletions (~12%) in these animals made up 98% of all alleles transmitted, the potential for additional expansions is thus limited in these animals. Nonetheless, since expansions and deletions totaled just 76% of transmitted alleles from *Atm*^{+/+} males, the 40% increase in expansions in the progeny of *Atm*^{+/-} fathers translates into an 88% decrease in the percentage of error-free transmissions (Figure 1C).

While *Atm*^{+/+} mothers produced progeny with expansions 37% of the time that the premutation allele was transmitted, *Atm*^{+/-} mothers produced expansions 55% of the time (Figure 1). Male offspring of *Atm*^{+/-}

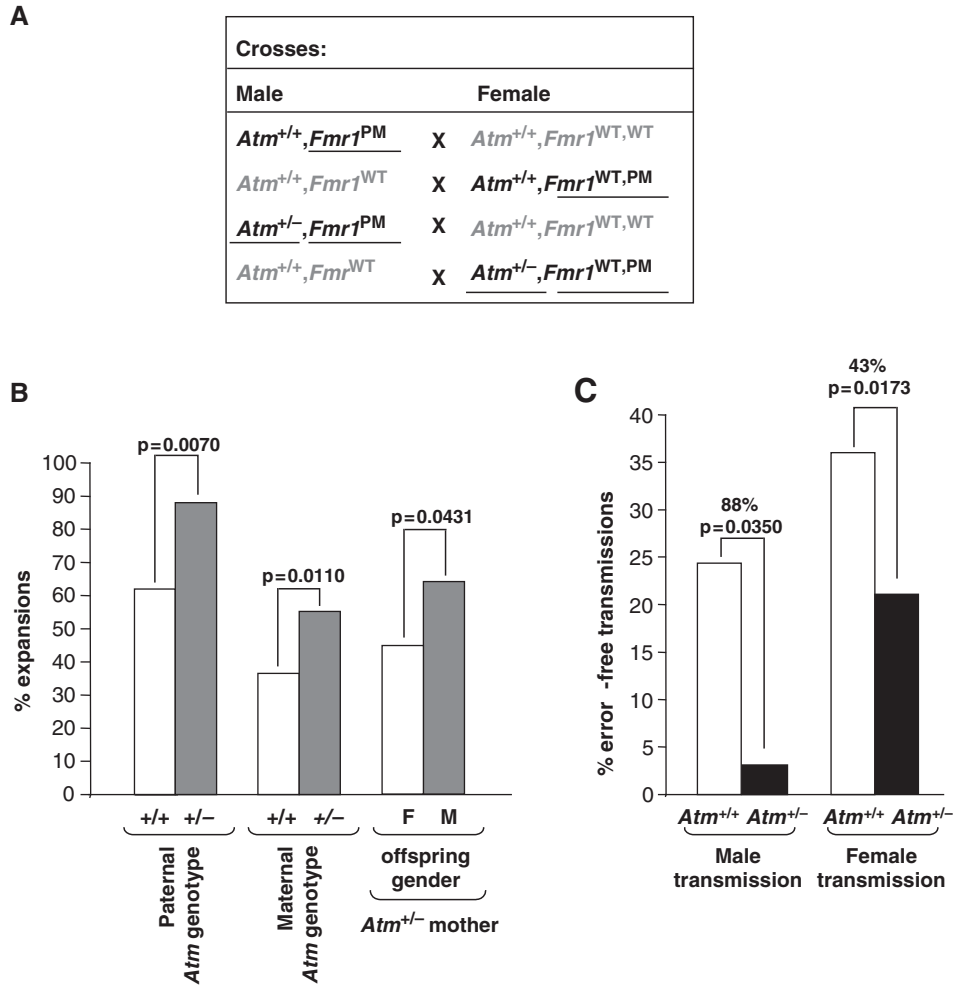


Figure 1. Intergenerational instability of the FX premutation allele in *Atm*^{+/-} and WT mice. (A) The crosses used in this study. All crosses involved one partner that was WT for both *Atm* and the *Fmr1* gene (shown in grey font) and one partner that was either WT or heterozygous for *Atm* and either hemizygous (males) or heterozygous for the *Fmr1* premutation allele (*Fmr1*^{PM}) as shown in the black font. The mutant alleles in each cross are underlined. The expansion frequencies (B) and the percentage of error-free transmissions (C) resulting from the crosses shown in (A) were determined as described in the ‘Materials and Methods’ section. The percent change in error-free transmission and the statistical significance of these changes, as determined by Fisher’s exact test, are shown.

mothers showed an expansion frequency that was significantly higher than that of their female littermates (64% compared with 45%; *P* = 0.0431). This suggests an effect of offspring gender on expansion rate, reminiscent of that reported for paternal transmission of CAG•CTG-repeats in a mouse model of Huntington Disease in which the animals are *Atm*^{+/-} (29,30). The effect of offspring gender in the FX premutation mice is not related to skewed transmission of the mutant *Atm* allele since the difference in expansion frequency did not reach statistical significance in *Atm*^{+/+} and *Atm*^{+/-} progeny irrespective of whether the premutation allele was maternally or paternally transmitted (data not shown). The effect of offspring gender could not be studied for paternal transmissions since the paternal X chromosome is not transmitted to male offspring.

The distribution of repeat length changes also differed in the progeny of these crosses (Figure 2). However, this change in the distribution was due primarily to an

increase in the number and size of the expansions since an *Atm* deficiency had no significant effect on the deletion frequency. This frequency was 27 and 24% for *Atm*^{+/+} and *Atm*^{+/-} mothers and 14 and 12% for *Atm*^{+/+} and *Atm*^{+/-} fathers, respectively (Figure 2). The fact that the expansion frequency increases in *Atm* heterozygous animals but the deletion frequency does not, supports the idea that expansions and deletions occur via different mechanisms as previously suggested (23,24).

The mean number of repeats added to each expanded allele was ~1.6-fold higher on transmission from an *Atm*^{+/-} parent than from an *Atm*^{+/+} parent (*P* = 0.0013 and 0.0006 for paternal and maternal transmissions, respectively, Figure 3). However, no difference was seen in the mean number of repeats added per expansion in male and female offspring of *Atm*^{+/-} mothers (Figure 3B). Thus, although the expansion frequency was higher in males, the number of repeats added with each expansion was similar.

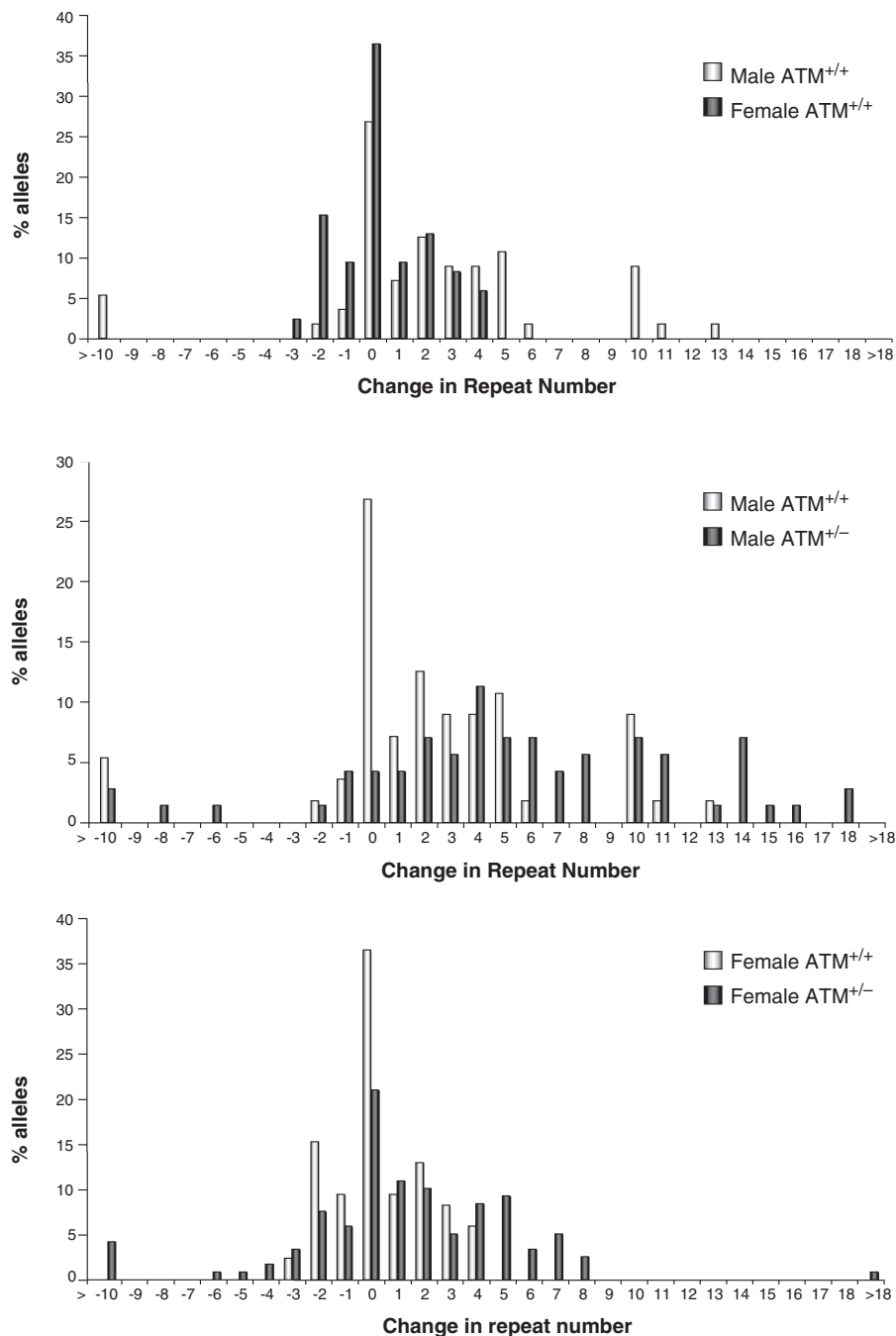


Figure 2. The distribution of repeat length changes on paternal and maternal transmission in WT and $Atm^{+/-}$ mice. The repeat length changes resulting from the various crosses shown in Figure 1A were determined as described in the 'Materials and Methods' section. The total number of alleles of each size class was then plotted as a percentage of the total number of alleles examined. Top panel: Effect of gender on the distribution of repeat length changes occurring on paternal and maternal transmission of the premutation allele in parents WT for Atm . Middle panel: Effect of Atm genotype on the repeat length changes occurring on paternal transmission of the premutation allele. Bottom panel: Effect of Atm genotype on the distribution of repeat length changes occurring on maternal transmission of the premutation allele.

Most Atm -null mice succumb to thymic lymphoma by ~4 months of age (27) thus limiting the age of Atm -null mice we could examine for instability. No evidence of repeat length changes were seen in the brain, heart, liver, kidney, testes or tail DNA from a 3-month-old male with 142 repeats (Figure 4A) and one with 153 repeats (Figure 4B). No difference was seen in the somatic instability of $Atm^{+/+}$ and $Atm^{+/-}$ animals at 1 year of

age either (data not shown). This suggests that the effect of Atm heterozygosity on repeat expansion is limited to germline events rather than events occurring prior to germline establishment in the parent or in the post-zygotic embryo. Furthermore, it also suggests that the ATM pathway does not contribute to the somatic instability that is seen in $Atm^{+/+}$ FX premutation mice.

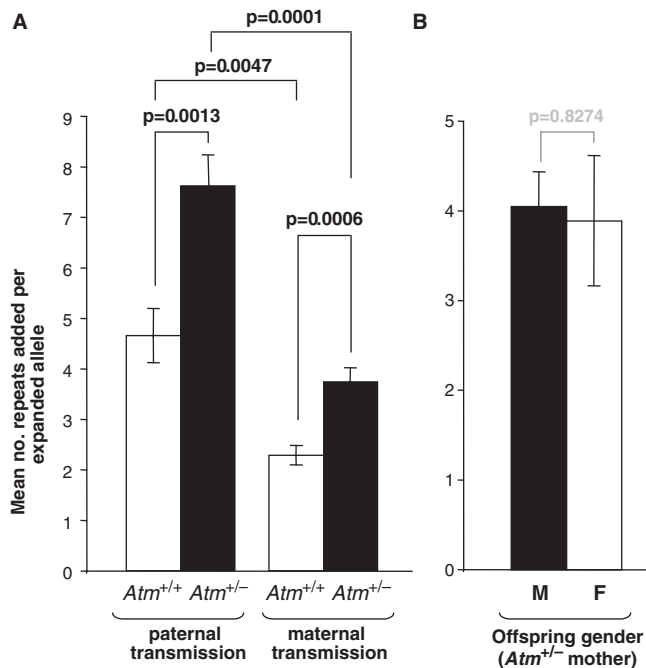


Figure 3. The effect of *Atm* heterozygosity of the offspring on the mean increase in repeat number per expansion event. The mean increase in repeat number seen for the expanded alleles and the standard error of the mean was determined for the offspring of the crosses shown in Figure 1A, as described in the 'Materials and Methods' section. The statistical significance of the difference in the means was determined by Student's *t*-test. (A) The mean increase in repeat number on transmission from *Atm*^{+/+} and *Atm*^{+/-} sires and dams. In the case of the maternal transmissions, the data from the male and female offspring are combined. (B) The mean increase in repeat number in male and female offspring of an *Atm*^{+/-} female.

DISCUSSION

We have shown that *Atm* heterozygosity results in a significant decrease in the rate of error-free transmission of the FX premutation allele in mice. This decrease is due primarily to an increase in the frequency of expansions since the frequency of deletions is not affected by *Atm* heterozygosity (Figure 1). In addition to an increase in the expansion frequency, the size of each expansion also increases significantly (Figures 2 and 3). Since in humans an increase in repeat number is associated both with an increased risk of pathology in premutation carriers and an increased risk of further expansions, the ATM-deficiency effectively increases the mutational burden generated by the repeat, by increasing both the likelihood that an expansion will occur and that the expanded allele will be deleterious. The effect of *Atm* heterozygosity on expansion frequency is more marked when the PM allele is paternally transmitted and in male offspring of females heterozygous for *Atm*.

Since the difference between the expansion frequency in *Atm*^{+/+} and *Atm*^{+/-} offspring of *Atm*^{+/-} mothers does not reach statistical significance, we suggest that the ATM-sensitive event that gives rise to expansions occurs most frequently early in gametogenesis when cells are diploid. The higher rate of expansions seen on

paternal transmission could either be related to a higher incidence of the ATM-sensitive event during spermatogenesis or to more efficient repair during oogenesis.

How then can the higher expansion frequency in male offspring of *Atm*^{+/-} mothers be explained? In the case of a mouse model for HD containing long CAG•CTG-repeats, it has been suggested that the gender differences represent 'a new kind of imprinting that depends on signals in the embryo' (30). Alternatively it may be that, at some frequency, DNA damage arising in the diploid oocyte persists unrepaired into the zygote. In the zygote, this damage may be repaired in one of two ways. The first mechanism is ATM-independent and restores the original repeat number, while the second is more error-prone and gives rise to expansions. Our data suggest that the more accurate repair mechanism is more likely to be used when the embryo is female. This increased likelihood of error-free repair in females may be a consequence of the presence of two X chromosomes. It is possible that the normal X chromosome is used as a template for repair via homologous recombination. However, since the WT *Fmr1* allele only contains eight repeats, repair using the normal X seems unlikely to restore the unrepaired premutation allele to its original size. Furthermore, this would not explain the effect of embryo gender in the HD mouse model since the repeat-containing allele is located on an autosome (31).

It may be that the gender effect is related to the expression of an X-linked gene DNA repair gene that is expressed from both X chromosomes in the female zygote before X inactivation occurs. If this gene is one of the 5–15% of genes on the X chromosome that escape X inactivation (32,33), it may also be responsible for the increased frequency of expansions seen on paternal transmission. A number of DNA repair genes are located on the X chromosome. For example, SMC1 α , previously known as SMC1L1 or SMC1, is an X-linked gene that is involved in both ATM-dependent (34) and ATR-dependent (35) DNA repair. It escapes X inactivation in humans (33,36) and is expressed at significantly higher levels in mouse ovaries than testes (37). Work is in progress to evaluate the role of SMC1 α and other X-linked DNA repair genes in FX repeat instability.

Whatever the mechanism, our data suggest that ATM normally acts to protect the genome against one class of repeat expansions that occur in FX premutation mice. Since the ATM-sensitive expansions differ in many key respects from that seen in ATR-deficient animals (23), it lends support to our hypothesis that the ATM- and ATR-sensitive expansion mechanisms are quite different. Since the ATM-sensitive mechanism shows a paternal expansion bias, it may be responsible for the small changes in repeat length that are characteristic of paternal transmission of FX premutation alleles in humans (12). Our data also raises the possibility that variations in proteins involved the ATM response to DNA damage may affect expansion risk at least for smaller FX premutation alleles.

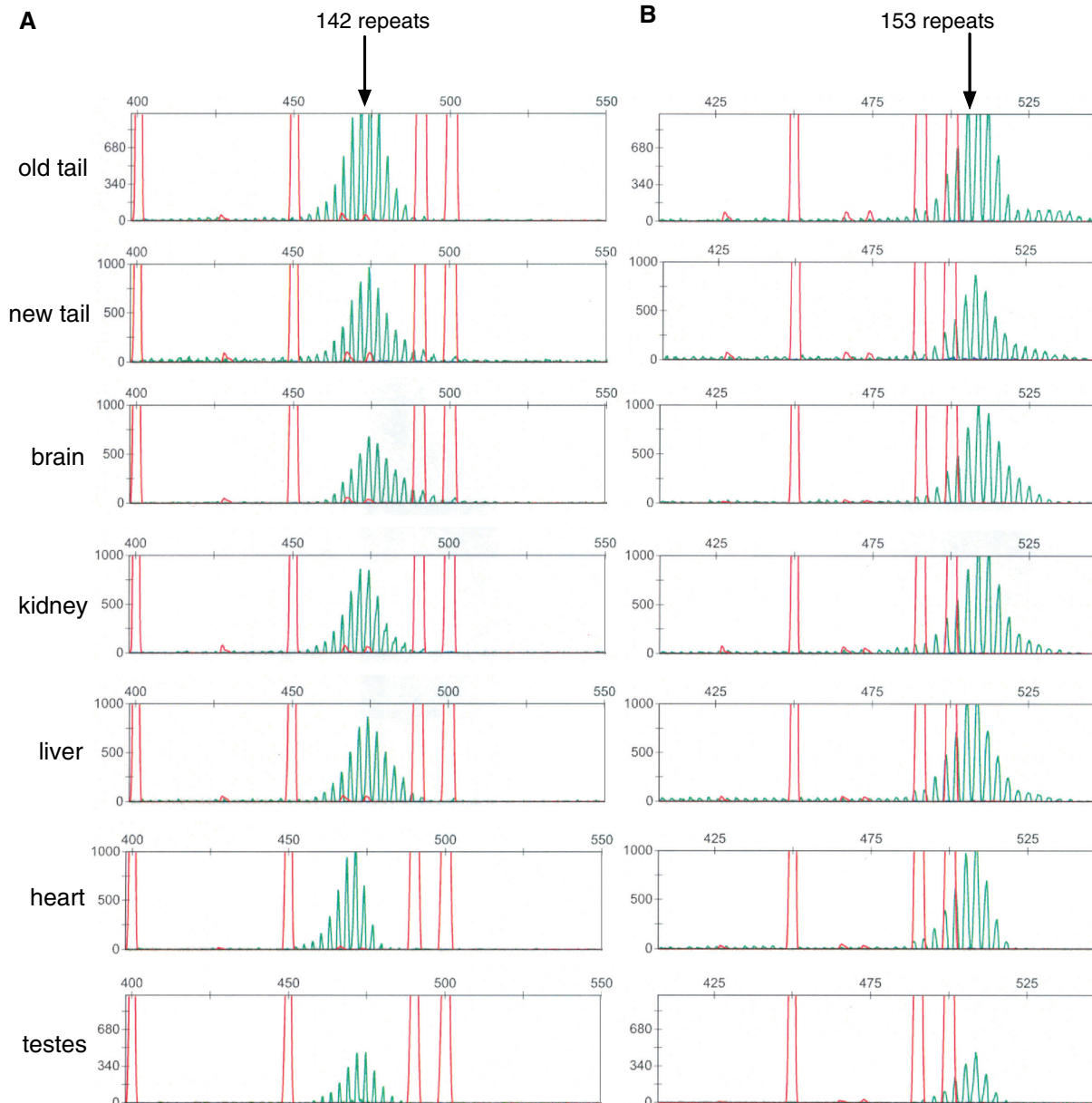


Figure 4. Somatic instability of the FX premutation allele in *Atm*-null mice. Fluorescent PCR analysis of the CGG•CCG-repeat tract size in different tissue of two *Atm*^{-/-} male mice at ~3 months of age. (A) Mouse with 142 repeats and (B) mouse with 153 repeats. Old-tail: tail snip taken at weaning, new-tail: tail sample taken after euthanasia.

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