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MSH2-Dependent Germinal CTG Repeat Expansions Are Produced Continuously in Spermatogonia from DM1 Transgenic Mice

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Myotonic dystrophy type 1 is a neuromuscular affection associated with the expansion of an unstable CTG repeat in the DM protein kinase gene. The disease is characterized by somatic tissue-specific mosaicism and very high intergenerational instability with a strong bias towards expansions. We used transgenic mice carrying more than 300 unstable CTG repeats within their large human genomic environment to investigate the dynamics of CTG repeat germinal mosaicism in males. Germinal mosaicism towards expansions was already present in spermatozoa at 7 weeks of age and continued to increase with age, suggesting that expansions are continuously produced throughout life. To determine the precise stage at which germinal expansions occur during spermatogenesis, we sorted and collected the different germ cell types produced during spermatogenesis from males of different ages and analyzed the CTG repeat mosaicism in each fraction. Strong mosaicisms towards expansions were already observed in spermatogonia before meiosis. In transgenic Msh2-deficient mice, germinal instability of the CTG repeats (only contractions) also occurs premeiotically. No significant difference in mosaicism was detected between spermatogonia and spermatozoa, arguing against continued expansions during postmeiotic stages. This indicates that germinal expansions are produced at the beginning of spermatogenesis, in spermatogonia, by a meiosis-independent mechanism involving MSH2.

Myotonic dystrophy type 1 (DM1) is associated with the expansion of a CTG trinucleotide repeat located in the 3′ untranslated region of the DM protein kinase (DMPK) gene at 19q13.3 (3, 7, 17, 27). In normal subjects, there are usually between 5 and 37 copies of this repeat, which remains stable following intergenerational transmissions. In DM1 patients, more than 50 CTG repeats are typically present, and the repeat is highly unstable and increases with each generation. The number of CTG repeats is positively correlated with the severity of symptoms and is negatively correlated with age at onset, resulting in an anticipation phenomenon that is particularly obvious in DM1 (19). The behavior of the CTG repeat between generations appears to depend on the sex of the transmitting parent. Paternal transmissions lead to larger expansions for <100 CTG repeats, whereas maternal transmissions lead to larger expansions when the CTG repeat tract contains >500 repeats in the transmitting parent (2, 24). In between, both paternal and maternal alleles expand. In addition to intergenerational instability, somatic CTG repeat-length mosaicism is also found in DM1 patients. Inter- and intratissue mosaicisms increasing with age are observed, with a strong bias towards expansions (1, 22).

DM1 is one of the growing group of diseases caused by dynamic mutations. This group currently comprises more than a dozen diseases, including Huntington's disease (HD), spinocerebellar ataxias, and fragile X syndrome, which are generally associated with CNG trinucleotide repeats (11, 46). The dynamics of the different trinucleotide repeats involved are very similar (intergenerational and somatic instability, bias towards expansions, CTG repeat number threshold for instability etc.), suggesting that the instability mechanisms are common to these diseases, especially those involving CTG or CAG tracts. Several hypotheses have been proposed to explain triplet repeat instability. These have been tested in bacteria, yeasts cell culture models, and transgenic mouse models. Expansions might occur during replication, through DNA polymerase pausing and/or slippage or FEN-1 endonuclease dysfunction in the CTG tract region (9, 20, 41–43, 45, 51). Furthermore, DNA repair mechanisms are clearly involved in instability, as the absence of MSH2, the main component of the mismatch repair pathway, leads in HD mice to the somatic stabilization of expanded repeats, showing that MSH2 is essential for the production of CAG expansions in tissues (29, 54a). Experiments using our DM300-328 transgenic mice, which carry >300 CTG repeats in their large (>45-kb) human genomic environment, revealed that the absence of MSH2 in this context does not stabilize the repeat but instead shifts the instability from expansions to contractions, not only in somatic tissues but also through generations (49). These results demonstrated that MSH2 is required for the formation of somatic and intergenerational CTG expansions. The CTG repeat is probably processed in a different way in the absence of MSH2, leading to intergenerational and somatic contractions. In a knock-in mouse model in which the 3′ part of the DMPK murine homolog is replaced by the corresponding human 3′ part of DMPK together with about 80 CTG repeats, van den Broek and collaborators observed a decrease of somatic CTG tract

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expansions in the absence of MSH3, one of the partners of MSH2 involved in mismatch repair and other mechanisms such as recombinational repair (54).

To determine the contribution of germinal mosaicism in the transmitting parent to intergenerational instability, several studies have been carried out on human patients with CAG repeat expansion diseases (HD, dentatorubropallidoluysian atrophy, spinocerebellar ataxia types 1 and 7, or fragile X syndrome) to look for correlations between the level of mosaicism in the sperm of the transmitting males and the size of the repeat inherited by their offspring. A high level of mosaicism was commonly observed in sperm, with this mosaicism overlapping the sizes inherited by the offspring, suggesting that intergenerational instability results mainly from germinal instability in the father (8, 12, 25, 34, 38, 52, 53). However, several studies, including one on fragile X fetuses and one on transmission in Friedreich ataxia families, suggested that intergenerational length changes could also involve a postzygotic instability event (13, 14, 37). In DM1 patients, a high level of CTG instability is observed in sperm from transmitting males, and this mosaicism more or less overlaps the CTG repeat sizes measured in blood from the offspring (22, 30).

A detailed analysis of CTG repeat-size variation between DM300-328 transgenic parents and their offspring carrying different MSH2 genotypes revealed that intergenerational instability probably results from the combination of two distinct events: germinal mosaicism in the transmitting parent and an MSH2-dependent instability event that takes place postzygotically, just after fertilization (49). Development analysis is required to elucidate the postzygotic instability event in our mice. Furthermore, the timing and dynamics of germinal and gametic instability in humans and in mouse models are still not fully understood. In HD mice carrying about 120 CAG repeats, male germinal expansions have been reported to be produced only in the latest stages of spermatogenesis (specifically during the transition from round spermatids to elongating spermatids) through a mechanism involving MSH2 (23, 28, 33). This is surprising, as the gene encoding MSH2 is not expressed during the final stages of mouse spermatogenesis and as no MSH2 protein can be detected in round spermatids in either men (5) or mice (47). In contrast, single-molecule DNA analysis of testicular germ cells from two HD patients suggested that expansions can occur before the end of the first meiotic division (55).

In several trinucleotide-repeat diseases and in our male transgenic mice, a positive correlation exists between the age of the transmitting parent and the repeat-length change in the offspring (48, 50). The evolution of parental gametic mosaicism throughout life may partly account for this association. A very small number of studies have measured repeat-size mosaicism in sperm from the same patient at different ages, but no clear increase was detected after 2 years in men with HD (25). However, the study periods may not be sufficiently long to detect changes in repeat-length mosaicism.

To elucidate the mechanism of germinal instability, we analyzed the status of CTG repeat instability in sperm at different ages to monitor the evolution of gametic mosaicism. By using the single-molecule PCR method, we demonstrated that a strong mosaicism towards expansions is present in spermatozoa, even very early during the mouse reproductive life. This mosaicism clearly increases with age, indicating that expansions are continuously produced through spermatogenesis in our mice. When assaying by normal or single-molecule PCR the levels of CTG repeat mosaicism in seven different germinal cell fractions sorted through a fluorescence-activated cell sorter (FACS), we found that mosaicism towards expansions was already present in spermatogonia. No differences were detected between mosaicisms in spermatogonia and spermatozoa. The presence of expansions in the first stages of spermatogenesis demonstrates that meiosis and postmeiotic mechanisms are probably not involved in germinal CTG repeat expansions. In $Msh2^{-/-}$ males, a majority of contractions were present in spermatogonia and no changes appeared in the following stages, indicating that MSH2 is necessary for the formation of germinal expansions in spermatogonia and that contraction events also occur before meiosis.

MATERIALS AND METHODS

Transgenic mice. We used the DM300-328 transgenic line carrying 45 kb of human genomic DNA cloned from a DM1 patient as described by Seznec et al. (50). These mice have been produced with B6D2/F1 fertilized mouse eggs and crossed over at least 15 generations with C57BL/6 mice. Transgenic status was assayed by PCR with the DMHR4 and DMHR5 primers (26). Tail DNA was amplified by PCR with primers 101 and 102, and CTG repeat size was measured after separating amplification products on a 4% acrylamide denaturing gel, as described previously (50).

Mouse dissection and blood and sperm DNA extraction. Mice were killed and dissected at 7 weeks, 11 months, or 28 months of age, in accordance with the French veterinary laws. Blood was collected from the heart just after death, and DNA was extracted by the phenol-chloroform method (26). Semen was collected from both vas deferens, and DNA was carefully extracted from spermatozoa to avoid contamination by DNA from other cell types (49, 50).

FACS of different germinal cell types. After dissection at 7 weeks or 11 months of age, both testes were collected and cleaned in 1× phosphate-buffered saline (PBS). After removal of the tunica albuginea, the seminiferous cords were unwound and rinsed in 1× PBS and then carefully minced to obtain the maximum amount of individual germ cells. After filtration through a 70-μm-pore-size cell strainer (Falcon), which retains all cellular aggregates, cells were counted and the cell solution was centrifuged for 10 min at 2,500 rpm (Jouan GR 422 instrument) at room temperature. The cell pellet was resuspended in cold 70% ethanol overnight at a final concentration of 5×10^6 cells/ml. The cell suspension was then centrifuged for 10 min at 2,500 rpm at room temperature and rinsed twice with fresh RPMI 1640 medium (GibcoBRL). RNA was removed by incubating for 30 min at room temperature with 500 μg of RNase A (Boehringer) per ml. Digitonin (10 μg/ml; Sigma) was then added and left for 2 min at 4°C in the dark to permeabilize the cells. Digitonin was removed by centrifugation at 2,500 rpm at room temperature for 10 min. The cell pellet was resuspended at a final concentration of 1.5×10^6 cells/ml in fresh RPMI 1640 with 200 nM MitoTracker GreenFM (Molecular Probes) for 30 min at 4°C in the dark. Propidium iodide (10 μg/ml; Sigma) was added 2 min before analyzing cell labeling with a FACS-Vantage cell sorter (Becton Dickinson). Cell doublets, apoptotic cells, and necrotic cells were discarded. The combination of the two labels allowed us to sort seven different germinal cell types (44). Each cell fraction was collected in fresh RPMI 1640 before DNA extraction. Cells from each fraction were mounted on microscope slides and analyzed with a fluorescence microscope (Leica) to assay the purity.

Extraction of DNA from FACS-sorted cells. Cell fractions were centrifuged for 10 min at 10,000 rpm (Sigma 1K15 instrument) at room temperature, and the resulting pellet was resuspended in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1% sodium dodecyl sulfate with 1 M β-mercaptoethanol for 1 h at room temperature. DNA was then extracted by the phenol-chloroform method.

Analysis of CTG repeat instability in the different germinal cell types. The CTG repeat region of each fraction was amplified with primers 101 and 102. Mosaicism was assayed by separating the amplification products on 3.5% acrylamide denaturing gels as previously described (49, 50).

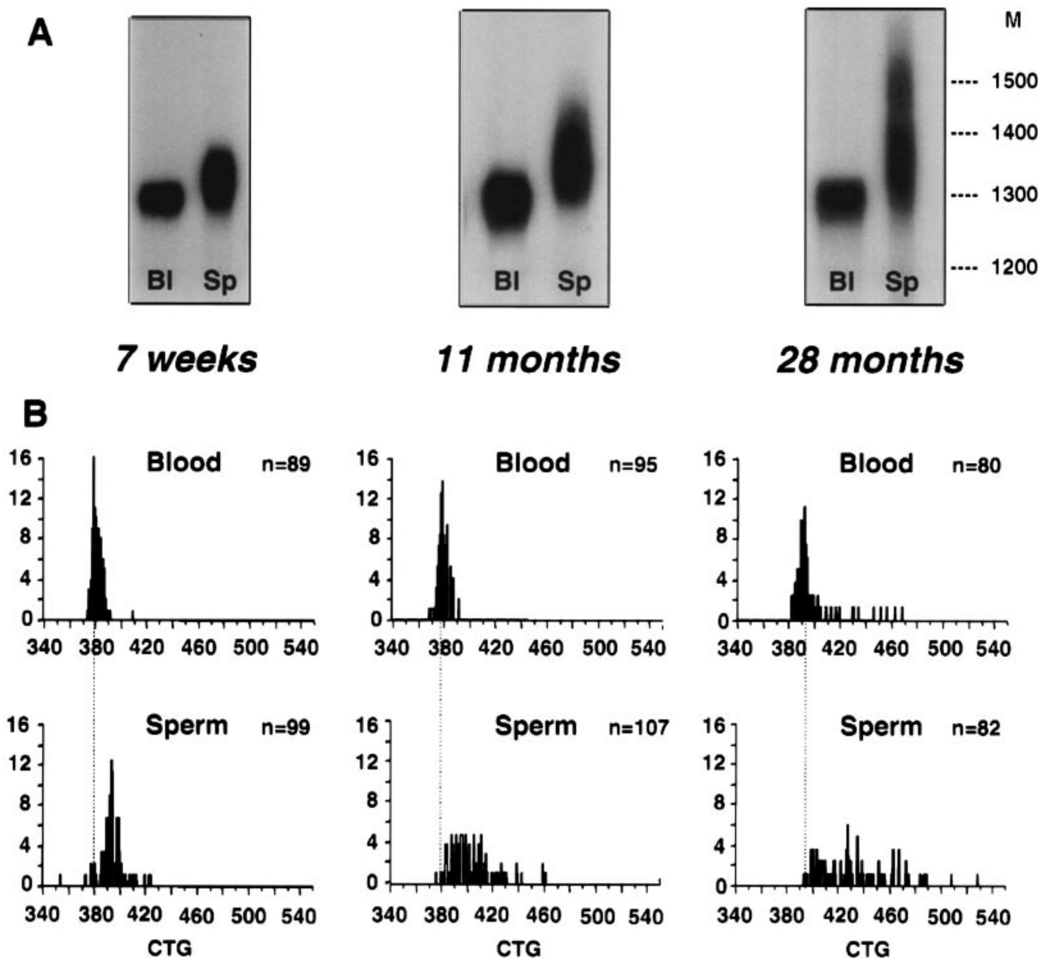

FIG. 1. Evolution of CTG repeat-length mosaicism in blood and sperm from transgenic mice throughout life. (A) CTG repeat-length mosaicism in blood (BL) and sperm (Sp) DNA from DM300-328 males at different ages, as measured by classical PCR. M, migration representation (in base pairs) of 100-bp DNA ladder. (B) Single-molecule PCR distributions of alleles with different CTG repeat lengths in blood and sperm DNA from DM300-328 transgenic males at different ages. The horizontal axis represents the CTG repeat size, whereas the vertical axis indicates the percentage of each size of allele with respect to all single genomes analyzed. n indicates the number of single genomes analyzed for each tissue. The dotted lines represent the number of CTG repeats measured in tail DNA at the time of weaning. CTG repeats in mice: 7-week-old males, 380; 11-month-old males, 378; 28-month-old males, 392.

PCR and single-molecule PCR. Blood, spermatozoa, and spermatogonia DNA was amplified by classical PCR and single-molecule PCR as previously described (35, 49).

Statistical analyses. Statistical analyses were performed with the StatView software by using the Mann-Whitney test (SAS Institute, Inc.).

RESULTS

CTG repeat mosaicism in spermatozoa throughout the reproductive life span. Transgenic DM300-328 males were dissected at 7 weeks, 11 months, and 28 months of age to monitor the CTG repeat-length mosaicism in the male germ line and to characterize the dynamics of germinal instability. We extracted DNA from blood and spermatozoa heads and used classical PCR and single-molecule PCR to determine the level of CTG repeat-length mosaicism. The CTG repeat-size mosaicism in blood and spermatozoa DNA was compared at each age and between the different ages to analyze their relative evolution. The CTG repeat remains relatively stable in the blood throughout life in our mice and in DM1 patients (49, 50), and thus the CTG repeat size determined from blood was used as a reference for CTG repeat-size mosaicism in other tissues. In mice, the appearance of the different germ cell types during development follows a time-dependent sequence. Spermatogonia differentiate from primordial germ cells and are the only cell type present at 7 days postpartum. While spermatogonia continue dividing, some of the germ cells enter meiosis and progress along Sertoli cells until the lumen of the seminiferous tubules. The first mature spermatozoa appear in the epididymis at 5.5 weeks after birth (31). Analysis of the mosaicism in spermatozoa of 7-week-old males allowed us to determine the influence of germ line development and the very early stages of spermatogenesis on CTG repeat mosaicism. In contrast, at 11 and 28 months of age, numerous rounds of spermatogenesis have already occurred and spermatogonia have already undergone numerous mitotic divisions.

Figure 1A shows typical results obtained after amplification

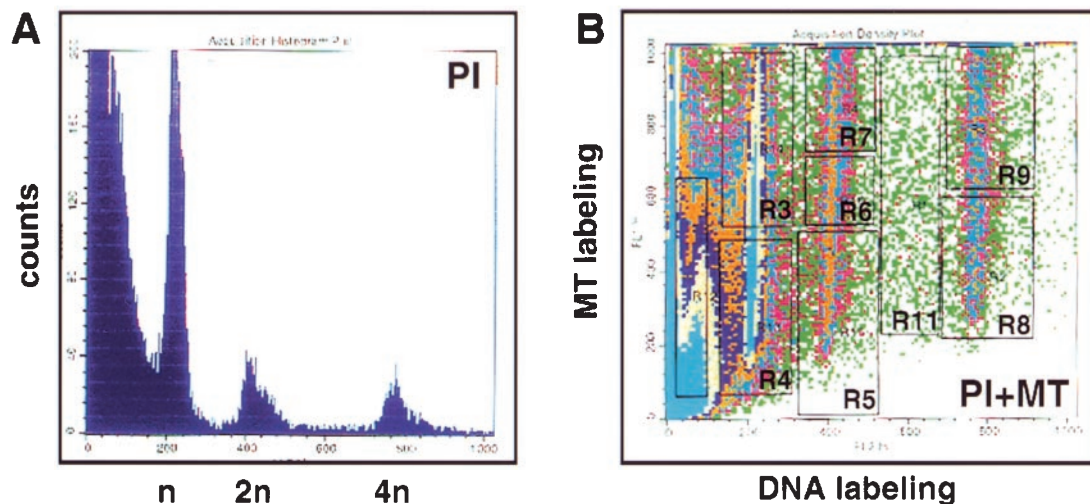

FIG. 2. Cellular labels used for the FACS of the different germinal cell types from transgenic mouse spermatogenesis. (A) The DNA content was measured by propidium iodide (PI) labeling. The horizontal axis represents fluorescence (arbitrary fluorescence units), and the vertical axis represents cell counts. (B) Double labeling against DNA (PI) (horizontal axis) and mitochondria (MT) (vertical axis). Sorting regions are indicated by black shapes. R3, round spermatids; R4, elongated spermatids; R5, spermatogonia; R6, preleptotene primary spermatocytes; R7, secondary spermatocytes; R8, = pachytene primary spermatocytes; R9, leptotene/zygotene primary spermatocytes; R11, cells in which DNA is replicating prior to the first meiotic division.

of the CTG repeat in blood and spermatozoa at the three studied ages by classical PCR with primers flanking the repeat. To ensure that the basal repeat size in each male had no effect on the level of somatic mosaicism, we studied only males that had equivalent numbers of CTG repeats in tail DNA upon weaning. Although no variation in the size of the CTG repeat was detectable in blood DNA, a distinct mosaicism that increased with age was observed in DNA from spermatozoa.

Single-molecule PCR with diluted DNA was then used to define more precisely the heterogeneity in the CTG repeat sizes (Fig. 1B). At 7 weeks, we could detect a very limited mosaicism in blood DNA, with very few variations around the major peak. In spermatozoa, the mosaicism was clearly detectable at this early stage, and CTG repeats already appeared to be longer than in blood ($P < 0.0001$), with a major peak 12 CTG repeats larger than in blood. At 11 months, the mosaicism in blood was still very weak. In contrast, the mosaicism in spermatozoa had increased significantly ($P < 0.0001$) and was clearly biased towards expansions, with a wider distribution of alleles of different sizes. The marked major peak disappeared, whereas large expansions could be observed (around +80 CTGs). At 28 months, some larger expansions could be observed in blood, but the mosaicism remained much more limited than in spermatozoa, in which the distribution of alleles of different lengths was much more widespread, with larger expansions reaching +140 CTGs. Thus, the CTG repeat-length mosaicism in spermatozoa increased considerably with age in our transgenic mice, with a strong bias towards expansions. Interestingly, in accordance with these results, we have previously found a positive correlation between the age of the transmitting male and the CTG repeat length inherited by the offspring (50). It should be noted that the CTG repeat lengths can vary slightly with age in blood. However, even at 28 months, most of the repeats were close to the initial size.

Dynamics of CTG repeat mosaicism throughout spermatogenesis. The evolution of mosaicism throughout spermatogenesis may make it possible to measure the influence of particular steps of this process on instability, such as mitotic divisions, induced recombination, and meiosis. To determine the precise stage of spermatogenesis at which the germinal CTG repeat expansions are produced, we used two labels (one for DNA and one for mitochondria) and FACS to collect seven cellular fractions corresponding to the different stages of spermatogenesis. We then analyzed the CTG repeat-length mosaicism in each fraction, in mature spermatozoa, and also in blood as a control. We used the DNA intercalating agent propidium iodide to separate different germ cell types according to their DNA content: n (round and elongated spermatids), 2n (spermatogonia, preleptotene primary spermatocytes, and secondary spermatocytes), and 4n (leptotene/zygotene and pachytene primary spermatocytes). Figure 2A shows the DNA content labeling of germ cell samples from an 11-month-old transgenic male. Three major peaks were obtained, corresponding to the different DNA contents encountered during spermatogenesis. However, this labeling alone is not sufficient to separate germ cells from different types carrying the same DNA content, such as spermatogonia and secondary spermatocytes or round and elongated spermatids. We therefore also used a fluorescent label directed against a mitochondrial membrane protein to reflect the activity and repartition of mitochondria throughout spermatogenesis (44). According to the stage of spermatogenesis, germinal cells need more or less energy to perform specific processes and mitochondria have different activities. The combination of these two complementary labels enabled us to sort seven different germinal cell types (Fig. 2B): spermatogonia (R5), preleptotene (R6), leptotene/zygotene (R9) and pachytene (R8) primary spermatocytes, secondary spermatocytes (R7), and round (R3) and elongated (R4) spermatids

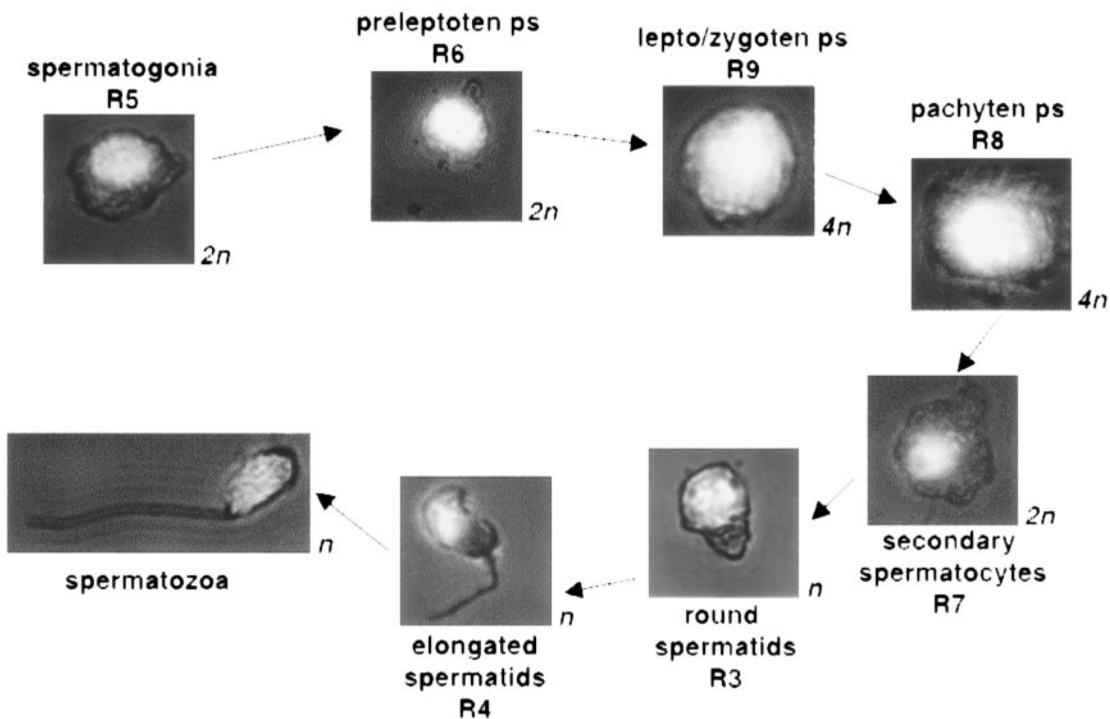

FIG. 3. Verification of cell fraction purity. Fluorescent microscopy (propidium iodide) slides were prepared for each sorted cellular fraction. Representative cells are shown. Arrows indicate the progression of mouse spermatogenesis from spermatogonia (top left) to spermatozoa (bottom left). The horizontal dotted line represents the first meiotic division, and the vertical dotted line represents the second meiotic division. ps, primary spermatocytes; lepto, leptotene. Diplotene primary spermatocytes were not sorted and are not represented here. n, 2n, and 4n indicate the relative DNA content at each stage of spermatogenesis.

(44). We ensured the purity of each cell type (estimated to be over 90%) by analyzing slides from each collected fraction by fluorescence microscopy (Fig. 3). After extraction of DNA from each cell fraction and also from blood and from mature spermatozoa, the level of CTG repeat-length mosaicism in each sample was measured. Interestingly, at 7 weeks of age, CTG repeat sizes appeared slightly larger in spermatogonia than in blood, with no detectable difference between spermatogonia and spermatozoa (Fig. 4). At 11 months of age, we observed marked mosaicism in spermatogonia, with larger CTG repeat sizes than in blood. In the following stages of spermatogenesis, including spermatozoa, mosaicism appeared to be equivalent to that observed in spermatogonia. The apparently faster migration products for round spermatids was not observed on repeated gels. The mosaicism was less pronounced at 7 weeks than at 11 months, showing that instability in spermatogonia increases with age in our transgenic mice. Although the separated seminiferous tubules were carefully washed in 1× PBS, some remaining testis interstitium cells such as Leydig cells (2n) may have been sorted together with spermatogonia (44). In adult mice, these cells represent 3.8% of the testicular volume, whereas the seminiferous tubules occupy 89.3% (36). Therefore, the proportion of Leydig cells remaining in the preparation and sorted with the spermatogonia is negligible. In addition, we observed a similar mosaicism pattern in preleptotene primary spermatocytes, the stage immediately after spermatogonia in spermatogenesis, which should not suffer from any contamination by somatic cells through these labels. These observations are consistent with mosaicism presented by the spermatogonia fraction being representative of instability in spermatogonia.

To determine whether CTG repeat-size mosaicism is equivalent in spermatogonia and spermatozoa, we used single-mol-

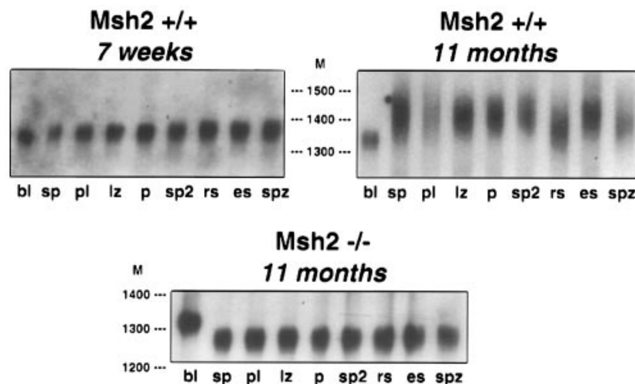

FIG. 4. CTG repeat-length mosaicism in blood, in spermatozoa, and in the different cellular fractions in $Msh2^{+/+}$ transgenic males at 7 weeks and 11 months of age and in an 11-month-old $Msh2^{-/-}$ transgenic male. Amplification products were separated on a 3.5% acrylamide denaturing gel and hybridized with a radiolabeled CAG probe. bl, blood; sp, spermatogonia; pl, preleptotene primary spermatocytes; lz, leptotene/zygotene primary spermatocytes; p, pachytene primary spermatocytes; sp2, secondary spermatocytes; rs, round spermatids; es, elongated spermatids; spz, spermatozoa. CTG repeat sizes in tail at weaning were as follows: $Msh2^{+/+}$ mice at 7 weeks, 401; $Msh2^{+/+}$ mice at 11 months, 406; $Msh2^{-/-}$ mouse at 11 months, 404. M, migration representation (in base pairs) of 100-bp DNA ladder.

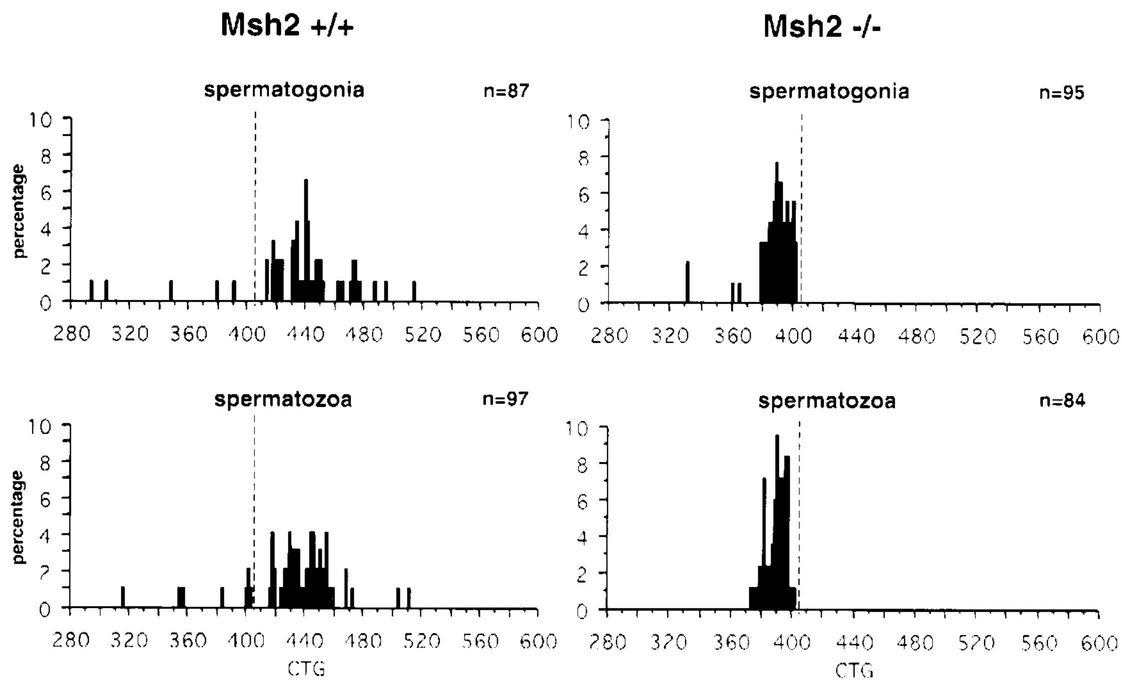

FIG. 5. Single-molecule distribution of alleles with different CTG repeat lengths in spermatogonia and spermatozoa from $Msh2^{+/+}$ and $Msh2^{-/-}$ transgenic males at 11 months of age. The horizontal axis represents the CTG repeat size, whereas the vertical axis indicates the percentage of each size of allele with respect to all single genomes analyzed. n indicates the number of single genomes analyzed for each tissue. The dotted line represents the number of CTG repeats measured in tail DNA at weaning. CTG repeats in mice: $Msh2^{+/+}$, 406; $Msh2^{-/-}$, 404.

ecule PCR to resolve the smears obtained for these two populations (Fig. 5). At 11 months of age, we observed a large CTG repeat-size mosaicism with a strong bias towards expansions in spermatogonia. Interestingly, in spermatozoa, the level of mosaicism was not statistically (by the Mann-Whitney test) different from that observed in spermatogonia. This suggests that in our mice, germinal expansions are already produced in spermatogonia (i.e., at the first stage of spermatogenesis) through a meiosis-independent mechanism.

We previously studied the effect of DNA repair genes on CTG repeat instability, and we have showed that the absence of MSH2 in transgenic mice shifted the instability from expansions to contractions in tissues and over generations (49). To determine whether the two types of CTG repeat-length change (expansions in $Msh2^{+/+}$ mice and contractions in $Msh2^{-/-}$ mice) are produced during the same time window, we separated the different germ line cell types from an $Msh2^{-/-}$ transgenic male at 11 months of age with a CTG repeat size in tail upon weaning similar to that of the $Msh2^{+/+}$ male. Classical PCR amplification of the CTG repeat detected contractions in spermatogonia (Fig. 4). Like for the $Msh2^{+/+}$ male, no significant difference could be noted between the levels of mosaicism in spermatogonia and in the subsequently cell types, including spermatozoa. This indicates that, similar to the expansion events in $Msh2^{+/+}$ mice, the deletion events can occur some time prior to but not after meiosis. While expansions were age dependent, the age dependency of the deletions has yet to be determined. Single-molecule PCR analyses performed on spermatogonia and spermatozoa from this $Msh2^{-/-}$ male confirmed that there was no statistically significant difference between spermatogonia and spermatozoa in the distribution of alleles with different CTG repeat lengths (Fig. 5). This suggests that germinal expansions in $Msh2^{+/+}$ males and germinal contractions in $Msh2^{-/-}$ males can both be produced in spermatogonia.

DISCUSSION

We investigated the evolution of the CTG repeat-length mosaicism in blood and sperm from transgenic males carrying >300 CTG repeats at different ages during their reproductive life span. Single-molecule PCR showed that expansions were already present in sperm at 7 weeks of age, just after male mice reach sexual maturity approximately 5.5 weeks after birth. Mosaicism in sperm increased with age, always with a strong bias towards expansions. The largest expansions were observed in sperm from the oldest males, which is consistent with the positive correlation between the age of the transmitting male and repeat sizes inherited by the offspring (50). In contrast, mosaicism in blood remained very weak at all stages, with only a few expansions being detected in the latest stages. At 7 weeks of age, the fact that the mean size of the CTG repeat was larger in spermatozoa than in blood can be explained by two nonexclusive hypotheses. The larger CTG repeat sizes observed in spermatozoa at this age may have been produced during germ line development or during the very early rounds of spermatogenesis, the first of which is completed at around 34 or 35 days after birth when the first mature spermatozoa appear in the epididymis (47). The fact that the germinal mosaicism increases with age suggests that expansions are continuously produced during spermatogenesis and that additive events are responsible for the high level of mosaicism observed in elderly

males. In the male germ line, different types of repair occur depending on the stage of spermatogenesis. During germ line development and thereafter, DNA replication repair occurs in both stem and premeiotic cells (4). Furthermore, germinal cells entering meiosis are subjected to several molecular events, such as chromosome pairing and induced recombination, and to different types of DNA repair (32).

We also studied the timing of CTG repeat instability throughout spermatogenesis, to determine the precise stage at which expansions are produced, especially with regard to meiosis, i.e., before, during, or after meiosis. This information should help us to understand the mechanism(s) involved in the production of expansions. We studied CTG repeat instability in spermatogonia, which are renewed and which proliferate during spermatogenesis in adult males; in primary spermatocytes (preleptotene, leptotene/zygotene, and pachytene), in which several molecular events successively occur (induced recombination, chromosome pairing, and DNA synthesis); in secondary spermatocytes, which are subject to the first meiotic division; and in round and elongated spermatids, which undergo major morphological changes and chromatin compaction before finally becoming spermatozoa. In yeast, the instability of CAG/CTG repeats increases during meiosis, mainly due to deletions (10, 21). Once cells enter meiosis, they may undergo molecular mechanisms such as repair associated with homologous recombination, taking place after self-induction of double-strand breaks in an SPO11-dependent manner (21). However, our data show that in our transgenic mice, germinal expansions are produced in spermatogonia and the length of the CTG repeat does not change between spermatogonia and mature spermatozoa. No meiosis-specific mechanisms appear to be necessary for expansions to occur during spermatogenesis in our transgenic mice. Our data suggest that germinal instability results from a mechanism that occurs during spermatogonia, therefore excluding the repair of self-induced double-strand breaks and homologous recombination during meiosis. The increase in mosaicism with age in the DM300-328 males probably results from the accumulation of expansions over lifetime, due to spermatogonia undergoing mitotic divisions and/or the repeated action of DNA repair mechanisms on these cells.

We recently studied the influence of DNA repair on CTG repeat instability by crossing our transgenic mice with mice having knockouts of genes belonging to the different DNA repair pathways, including the Msh2 mismatch repair pathway. We did not observe any stabilization of the repeat in transgenic $Msh2^{-/-}$ mice, unlike in another transgenic mice model (23, 29). In contrast, in the absence of MSH2, instability was shifted from expansions to contractions, both across generations and in tissues including spermatozoa (49). It is still unclear whether expansions in $Msh2^{+/+}$ mice and contractions in $Msh2^{-/-}$ mice are produced by the same mechanism, with the direction of the instability depending on the presence of MSH2, or whether a totally different mechanism processes the CTG repeat in the absence of MSH2, leading to contractions instead of expansions. We decided to determine whether expansions in $Msh2^{+/+}$ males and contractions in $Msh2^{-/-}$ males are observed at the same stage of spermatogenesis, in order to obtain further insight into the mechanism(s) involved in both types of change in CTG repeat length. We therefore monitored the CTG repeat contractions throughout spermatogenesis in $Msh2^{-/-}$ males and observed that contractions were already present in spermatogonia, with a length mosaicism similar to that observed in spermatozoa. The role of two partners of MSH2 in somatic instability was recently investigated in knock-in mice carrying the last exons of the human DMPK gene with 80 CTGs (54). The absence of MSH3 leads to the loss of somatic expansions, suggesting that MSH3, like MSH2, is involved in the somatic expansions of CTG repeats in mice. During mouse spermatogenesis, Msh2 is usually expressed to higher levels in spermatogonia and early primary spermatocytes than in other germinal cell types or tissues, whereas the expression of Msh3 is very weak in spermatogonia but peaks in early primary spermatocytes (preleptotene and leptotene) (47). MSH2 is crucial for expansions in spermatogonia, and MSH3 is probably important in germinal instability given its involvement in somatic mosaicism. However, as the level of expression of Msh3 is very low in spermatogonia, the stage where expansions occur in our mice, it is important to determine whether the absence of MSH3, a potential partner of MSH2 in the mechanisms generating expansions, modifies germinal instability. The formation of contractions observed in the absence of MSH2 remains limited to spermatogonia in our mice, as no further deletions were observed in the following stages of spermatogenesis. We showed that contractions and MSH2-dependent expansions are probably produced at the same stage of spermatogenesis in DM300-328 mice. However, this does not imply that expansions and contractions result from the same repair pathways, and the proteins involved in these events remain to be identified.

A role for MSH2 in the generation of germinal expansions via gap repair was previously proposed by Kovtun and McMurray (23). In mice carrying about 120 CAG repeats, expansions during spermatogenesis seem to be limited to the latest stages of spermatogenesis, with no mosaicism detectable in the earlier stages, including spermatogonia, whereas instability appears to occur before meiosis in human HD patients (55). When Msh2 is missing in these transgenic mice, no expansions occur in spermatozoa and the CAG repeat is stabilized, suggesting that germinal expansions are produced in spermatozoa by an Msh2-dependent mechanism. After the completion of meiosis, postmeiotic repair might occur in spermatids to ensure the DNA integrity of future gametes. However, at this step, the expression levels of many repair genes and genes encoding enzymes such as MSH2, MSH3, or PMS2 dramatically decrease, eventually becoming undetectable in elongated spermatids (47). In contrast, in our transgenic mice, germinal expansions appear to be generated in spermatogonia, closely matching what was reported for human HD patients (55). In addition, the absence of MSH2 shifts instability from expansions to contractions. One of the first hypotheses to explain these differences in the dynamics and timing of trinucleotide repeat instability is the different genomic contexts surrounding the repeats in these models, as it has already been suggested that they affect the direction and range of repeat-length changes (6, 9, 18, 26a, 50, 56). Furthermore, the frequency and size of intergenerational repeat expansions were very high in our mice. The different technical approaches could also partly explain the differences. We collected spermatozoa from the vas deferens and then extracted DNA from spermatozoa heads

rather than from epididymis sperm. We have previously observed that DNA from epididymal cells (where instability of the repeat is very high, especially in the cauda epidydimis [data not shown]) can contaminate the DNA extracted from sperm without a specific protocol. By combining mitochondrial and DNA labelings, we were able to distinguish seven cell types found during spermatogenesis, whereas only three can be distinguished when only DNA content and cell size are analyzed. The range of trinucleotide repeat-length changes also differs, as does the method used to measure them in the two cases. We used single-molecule PCR on a large set of DNA samples containing single genomes. This is a very powerful tool for resolving the mosaic smears obtained after classical PCR amplification of trinucleotide repeats (35). We were able to detect a broad range of expansions in spermatogonia and spermatozoa, from +1 to +150 CTGs with a mean of about +30 CTGs. These expansions were clearly distinguishable from the PCR stutter classically observed during triplet-repeat amplification, due to polymerase slippage. This overcomes the problem of small expansions and contractions when the DNA input is too great, as discussed by Zhang et al. (56).

Our results imply that CTG repeat-length mosaicism is already present in germinal cells prior to meiosis and that the mechanism responsible for generating expansions is meiosis independent, occurs continuously throughout life, and involves MSH2. This limits the number of molecular mechanisms that can generate expansions. Interestingly, in addition to their role in mismatch repair, MSH2 and MSH3 are also involved in the repair of double-strand breaks by homologous recombination (15). MSH2 and MSH3 together ensure the homology of the sequence used as a template for this type of repair and scan this template for mismatches, small insertions, and deletions. Therefore, this process does not seem to be involved in the production of expansions in our mice, as shown by our previous results with Rad52 and Rad54, both of which are major actors in this repair pathway but which have no effect on the frequency of CTG repeat expansions (49). MSH2 also participates in single-strand annealing (SSA), which is able to repair double-strand breaks but does not involve classical double-strand break repair proteins like Rad51 or Rad54 (40). It would be interesting to study the effects of the ERRC1 and ERCC4 proteins (encoded by the mammalian homologs of the *Saccharomyces cerevisiae* Rad10 and Rad1 genes, respectively) on CTG repeat-size mosaicism, as these proteins, together with MSH2, are crucial for the ability of SSA to correct DNA damage (39). As trinucleotide repeat tracts are length-dependent breaking sites in yeast (16), the repeated action of the SSA pathway on these particular sequences may be the factor leading to repeat instability in some tissues. In all cases, the occurrence of germinal expansion events in spermatogonia, which are mitotically dividing 2n germ cells, suggests that the mechanisms responsible for instability in germ line and somatic tissues are potentially identical.

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