CTG repeat instability and size variation timing in DNA repair-deficient mice

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**Type 1 myotonic dystrophy is caused by the expansion
of an unstable CTG repeat in the DMPK gene. We
have investigated the molecular mechanisms under-
lying the CTG repeat instability by crossing trans-
genic mice carrying >300 unstable CTG repeats in
their human chromatin environment with mice knock-
out for genes involved in various DNA repair path-
ways: *Msh2* (mismatch repair), *Rad52* and *Rad54*
(homologous recombination) and *DNA-PKcs* (non-
homologous end-joining). Genes of the non-homolo-
gous end-joining and homologous recombination path-
ways did not seem to affect repeat instability. Only
lack of Rad52 led to a slight decrease in expansion
range. Unexpectedly, the absence of Msh2 did not
result in stabilization of the CTG repeats in our
model. Instead, it shifted the instability towards con-
tractions rather than expansions, both in tissues and
through generations. Furthermore, we carefully ana-
lyzed repeat transmissions with different *Msh2* geno-
types to determine the timing of intergenerational
instability. We found that instability over generations
depends not only on parental germinal instability, but
also on a second event taking place after fertilization.**
Keywords: CTG instability/double-strand break repair/
mismatch repair/transgenic mice

Introduction

Type 1 myotonic dystrophy (DM1) is a neuromuscular
condition involving myotonia and progressive muscle
wasting. Digestive and respiratory functions are also
affected, as are the central nervous system and the eyes
(Harper, 2001). DM1 results from the expansion of an
unstable CTG repeat in the 3′ untranslated region of the
DM protein kinase gene (*DMPK*) (Aslanidis *et al.*, 1992;
Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*,
1992). In unaffected individuals, there are 5–37 CTG
repeats in this gene, and repeat size remains stable over
generations. In DM1 patients, a high level of intergenera-
tional instability is observed, with a strong bias towards
expansions. The number of CTG repeats is >50 and
increases from generation to generation, to several thou-
sand repeats. The sex and CTG repeat size of the
transmitting parent affect CTG size in the offspring
(Lavedan *et al.*, 1993; Ashizawa *et al.*, 1994). In DM1
families, the size of the CTG repeat is generally correlated
with the severity of the symptoms and age at onset,
accounting for the anticipation phenomenon observed
from generation to generation (Harper *et al.*, 1992).
Somatic CTG repeat instability is observed in various
tissues from DM1 patients, with a strong bias towards
expansions. This instability probably appears early in
embryogenesis, increases after 16 weeks of gestation and
continues into adulthood (Martorell *et al.*, 1997).

Since the discovery of dynamic mutations, many studies
have focused on the molecular mechanisms of instability.
The hairpins and slipped-strand structures observed
in vitro with trinucleotide arrays are strongly suspected
to induce instability through their interference with
replication processes (Gacy *et al.*, 1995; Samadashwily
et al., 1997; Moore *et al.*, 1999; Pearson *et al.*, 2002).
These structures may cause DNA polymerase to pause
while replicating a triplet repeat, resulting in its dis-
sociation from the newly synthesized strand (Ohshima and
Wells, 1997; Samadashwily *et al.*, 1997; Viguera *et al.*,
2001). This strand would then separate from its template
and might reassociate elsewhere within the repeat, leading
to expansions on the resumption of replication, a phe-
nomenon known as replication slippage (Pearson and
Sinden, 1998; Petruska *et al.*, 1998). In addition, replica-
tion-associated DNA hairpins arising at triplet repeat
sequences also seem to inhibit the FEN-1 structure-
specific endonuclease and may contribute to instability at
the triplet repeat sequence (Spiro *et al.*, 1999; Henricksen
et al., 2000). The absence of FEN-1 due to the disruption
of the yeast *RAD27* gene also leads to destabilization of
minisatellites, microsatellites and triplet repeats. DNA
breakage within the region of CTG repeats may increase
the action of DNA repair mechanisms at this particular
site (Freudenreich *et al.*, 1998; Kokoska *et al.*, 1998;
Schumacher *et al.*, 1998). Furthermore, the direction of
replication and the distance between the origin of repli-
cation and the repeat tract determine whether instability
tends towards expansions or contractions *in vitro* and in
primate cells (Cleary *et al.*, 2002; Panigrahi *et al.*, 2002).
However, analyses of transgenic mice reproducing CTG
somatic instability have shown that there is no correlation
between somatic mosaicism and tissue division rate,
suggesting the involvement of replication-independent
mechanisms in non-dividing cells (Lia *et al.*, 1998).
Therefore, one or several DNA repair pathways, linked or
not to replication, may also be involved in triplet repeat
instability.

Double-strand break (DSB) repair involving homologous recombination (DSBR-HR) affects the stability of CAG and CTG repeats in yeast, leading to both expansions and contractions (Richard *et al.*, 1999; Jankowski *et al.*, 2000; Karran, 2000). HR is mediated by the Rad52 group of proteins, including Rad52 and Rad54 (van Gent *et al.*, 2001). DSB repair can also occur via the non-homologous end-joining mechanism (NHEJ), which requires little, if any, sequence homology (Haber, 2000). Mismatch repair (MMR) has been demonstrated to be involved in triplet repeat instability, based on studies of the main component of this pathway, *MutS* in bacteria and its homolog, *Msh2* in yeast and mammals. The absence of MutS in *Escherichia coli* results in stabilization of 64, 130 or 180 CTGs, and prevention of the large deletions that occur in the presence of MutS (Jaworski *et al.*, 1995; Schumacher *et al.*, 1998). In the yeast *Saccharomyces cerevisiae*, CTG and CAG repeats with 50 units display large natural deletions and do not seem to be affected by *Msh2* mutations, indicating that Msh2 is not involved in such large deletions (Miret *et al.*, 1997). Human Msh2 is known to bind tightly to the secondary structures formed by trinucleotide repeats *in vitro* and this binding may activate repair mechanisms (Pearson *et al.*, 1997). In a transgenic mouse model for Huntington's disease (HD) with 130 CAG units, somatic expansions disappear if *Msh2* is deleted, indicating that Msh2 is involved in the generation of somatic expansions (Mangiarini *et al.*, 1997; Manley *et al.*, 1999). Furthermore, in these mice, Msh2 has been proposed to be involved in gap repair generating instability of CAG repeats in spermatozoa (Kovtun and McMurray, 2001). Studies of the partners of Msh2 in MMR: Msh3 and Msh6, in a DM1 knock-in mouse model carrying 84 CTGs have shown that the absence of Msh3 abolishes somatic expansions whereas the absence of Msh6 leads only to a slight increase in somatic expansions (van den Broek *et al.*, 2002).

We decided to investigate the involvement of various DNA repair pathways in CTG repeat instability, using our DM1 transgenic mouse model, which carries a large repeat (>300 CTGs) embedded in its human chromatin environment of >45 kb. The use of long flanking sequences from the human genome and of a large expansion have facilitated faithful reproduction of the dynamics of the CTG repeat observed in DM1 patients, both intergenerationally and somatically (Gourdon *et al.*, 1997b; Seznec *et al.*, 2000). As observed in DM1 families, these mice display >90% CTG repeat length variation over generations, with a strong bias towards expansions (>85%). Parental age, repeat size and sex affect size variation. These mice display a high level of tissue-specific mosaicism biased towards expansions, which increases with age. Our model is therefore perfectly suitable for studying the influence of various DNA repair genes on intergenerational and somatic instability that cannot be approached in unicellular organisms. Analyses of CTG repeat size over generations and in tissues, in various DNA repair-deficient backgrounds, showed that a lack of expression of the *Rad54* (DSBR-HR) and DNA-dependent protein kinase catalytic subunit or *DNA-PKcs* (NHEJ) genes had no major effect on CTG instability. However, the absence of Rad52 (DSBR-HR) had no effect on the frequency of expansions and contractions, but did slightly decrease the size of expansions. Furthermore, in sharp contrast to the stabilization of a CAG trinucleotide repeat observed in transgenic mice deficient in *Msh2* (Manley *et al.*, 1999; Kovtun and McMurray, 2001), the absence of Msh2 in our transgenic mice did not result in the stabilization of CTG repeats. Instead, it changed instability from expansions to contractions. In *Msh2*-deficient transgenic mice, we observed almost exclusively contractions, both somatically and over successive generations. Moreover, analysis of transmissions in parents and offspring of different *Msh2* genotypes made it possible to determine the timing of intergenerational instability. We present data showing that intergenerational instability results not only from parental germinal instability, but also from a 'second event' taking place just after fertilization.

Results

Experimental strategy

Mice from the DM300–328 line, carrying >300 CTG repeats, were crossed with knockout mice for genes belonging to various DNA repair pathways. We assessed intergenerational instability over successive generations and the somatic instability of the CTG repeat in several tissues, in the various DNA repair-deficient backgrounds. After a series of matings, we obtained generations of transgenic DM300–328 mice lacking both alleles of the assayed gene. We determined the length of the CTG repeat of the transgenic transmitting parent and of the transgenic offspring by PCR, using tail DNA obtained at weaning (Seznec *et al.*, 2000). The change in CTG repeat length from generation to generation in the mutant lines was compared with that of controls wild-type for both alleles of the tested gene, and/or with mice +/– for the studied DNA repair genes.

Effect of the Rad52 and Rad54 genes on intergenerational CTG instability

For DSB repair via HR, it was possible to investigate the potential roles of two genes, *Rad52* and *Rad54*. Rad52 promotes HR by assisting Rad51, the key player in HR which is responsible for recognition of homologous DNA sequences and DNA strand exchange (Paques and Haber, 1999; Sugiyama and Kowalczykowski, 2002). The *Rad52* knockout mice are viable and fertile. *Rad52* knockout embryonic stem (ES) cells display a slightly reduced frequency of HR as measured by targeted homologous integration, but are not hypersensitive to DSB-inducing agents (Rijkers *et al.*, 1998; Whitehouse *et al.*, 1999). Rad54 also stimulates Rad51-mediated HR, most likely at a step after Rad52 function (Petukhova *et al.*, 1998). Rad54 predominantly promotes HR with the use of the sister chromatid as repair template (Arbel *et al.*, 1999; Dronkert *et al.*, 2000). *Rad54* knockout ES cells are sensitive to ionizing radiation and interstrand DNA crosslinking agents, display a reduced frequency of targeted homologous integration and are defective in DSB repair (Essers *et al.*, 1997; Dronkert *et al.*, 2000). Furthermore, *Rad54* knockout mice are sensitive to interstrand DNA crosslinking agents and ionizing radiation sensitive in combination with a DNA-PKcs mutation (Essers *et al.*, 1997, 2000).

Table I. Intergenerational instability in transgenic mice with different DNA repair-deficient backgrounds

Parent to offspring transmission	Transmissions analyzed	Expansion frequency (%)	Mean expansion length (CTG)	Contraction frequency (%)	Mean contraction length (CTG)	Unchanged repeat size frequency (%)
DM300–328	299	86.3	+14.5	8.7	–10.1	5.0
Rad52 –/– to –/–	68	89.7	+9.3	4.4	–12.7	5.9
Rad54 –/– to –/–	55	85.5	+13.3	10.9	–14.5	3.6
DNA-PKcs –/– to –/–	91	64.8	+7.6	27.5	–10.7	7.7
DNA-PKcs +/– to +/–	37	62.2	+10.0	27.0	–10.6	10.8
Msh2 –/– to –/–	82	4.9	+4.0	90.2	–14.5	4.9
Msh2 +/– to +/–	82	78.0	+12.8	19.5	–9.9	2.5

+/–, –/– represent specific genotypes at the murine locus for the assayed gene: +, wild-type allele; –, mutant allele.

As the *Rad52* and *Rad54* knockout mice were bred from mice with a genetic background identical to that of our transgenic DM300–328 mice (C57BL/6), we compared transgenic *Rad52* and *Rad54* knockout mice with DM300–328 mice. Table I presents the data of the various DNA repair-deficient transgenic lines, for male and female transmissions combined. As reported previously, CTG repeat in the DM300–328 mice showed a high level of intergenerational instability, with >90% size variation in the offspring, and a bias towards expansions (86.3%), with a mean size of +14.5 CTG. Contractions accounted for only 8.7% of total transmissions. In *Rad52* or *Rad54*-deficient mice, the frequency of mutations was unaffected and a strong bias towards expansions was observed (89.7% for *Rad52* –/–, 85.5% for *Rad54* –/–). Although the mean size of expansions was unchanged by the absence of Rad54 (+13.3 CTG), a significant (*P* <0.001) decrease was observed for *Rad52* –/– to –/– transmissions, with a mean of +9.3 CTG, as opposed to +14.5 for DM300–328 and +14.4 for *Rad52* +/– to +/– (not shown). We showed previously that variations of the CTG repeat length from generation to generation depend on the age and CTG repeat size of the transmitting parent. This could eventually introduce a bias in the results if transmissions are obtained from mice with different age and CTG repeat (Seznec *et al.*, 2000). We therefore also analyzed groups of transmitting mice with similar CTG repeat sizes and ages and obtained the same results (data not shown). Overall, our results show that the complete loss of *Rad52* and *Rad54* does not affect the mutability of the CTG repeat, or the frequency of expansions and contractions in our transgenic mice. However, loss of *Rad52* resulted in a significant decrease in the mean size of expansions.

DNA-PKcs has no effect on intergenerational CTG instability

We investigated the effect of the NHEJ repair pathway on CTG repeat instability with the use of a *DNA-PKcs* mutant. Together with the heterodimer of the Ku70 and Ku80 proteins, DNA-PKcs forms the DNA–PK complex. The complex can juxtapose broken ends and the DNA–PK complex bound to one end may trans-phosphorylate its counterpart on the other end. This could induce a change in the overall conformation of the complex, providing free access for repair proteins such as XRCC4 and ligase IV for repair termination (Haber, 2000). DNA-PKcs is also involved in the V(D)J recombination process, and mice with mutations in the gene encoding DNA-PKcs display a SCID (severe combined immunodeficiency) phenotype

(Taccioli *et al.*, 1998). These mice are viable and fertile despite also displaying radiosensitivity and defects in the NHEJ repair process.

The comparison of DNA-PKcs +/– to +/– and –/– to –/– transmissions showed no significant difference in mutation frequency or range (Table I). There was a bias towards expansions for both transmissions (62.2 and 64.8%, respectively), showing that loss of the remaining functional *DNA-PKcs* allele did not change intergenerational instability in our model. It has to be noted that both the frequency and mean size of expansions were lower in *DNA-PKcs* +/– to +/– transmissions than in DM300–328 (*P* = 0.0007, *P* = 0.04). As the absence of the last *DNA-PKcs* allele did not affect intergenerational instability (same level of instability in *DNA-PKcs* +/– and *DNA-PKcs* –/– transmissions), the differences observed between the *DNA-PKcs* and DM300–328 mice may be accounted for by the different genetic backgrounds of the two lines (CB17 and C57BL/6). Therefore, DNA-PKcs is probably not involved in the repeat instability mechanisms.

The loss of Msh2 does not affect the mutability of the repeat but drives instability from expansions towards contractions

For MMR, we studied the influence of Msh2 because of its central role in this process. It recognizes both true base–base mismatches (when complexed with Msh6, giving rise to the MutSα complex) and insertion/deletion loops of up to 12 bp (when complexed with Msh3, giving rise to the MutSβ complex) (Marti *et al.*, 2002). In both complexes, Msh2 is thought to act as a scaffold, with its two partners directly involved in DNA defect recognition, possibly in association with the PCNA sliding clamp (Bellacosa, 2001; Bowers *et al.*, 2001). This recognition induces a change in the conformation of the DNA helix at this site, resulting in activation of the MutLα and MutLβ complexes, and ATP/ADP exchanges trigger a cascade of events leading to repair (Marti *et al.*, 2002). In yeast, Msh2, in association with Msh3, ensures that there is a high level of sequence similarity between the invading 3′ ssDNA to be repaired and the matrix chosen as a template, but this protein is not involved in Holliday junction resolution. The knockout mice used in our study had very low MMR efficiency, with microsatellite instability, hyper-recombination and a predisposition to lymphoma. They were, however, viable and fertile (de Wind *et al.*, 1995).

The results obtained with mice transgenic for the repeat and carrying different *Msh2* genotypes were more striking
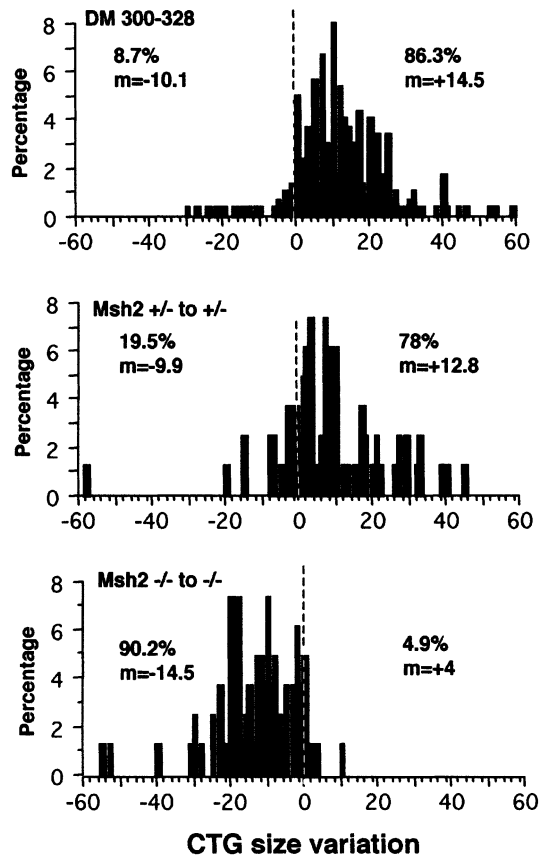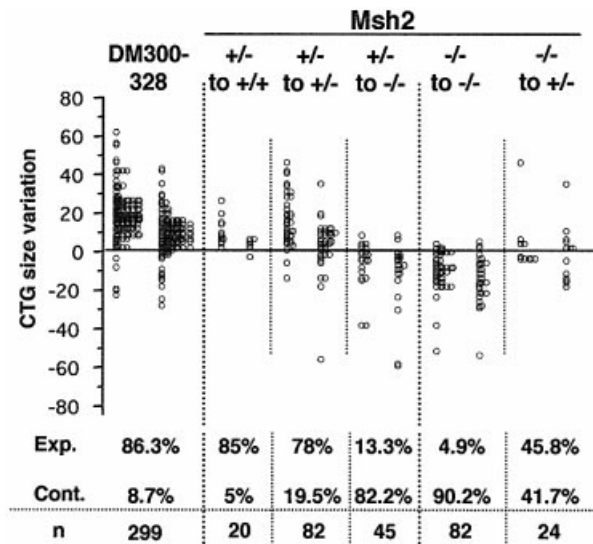
Fig. 1. Changes in CTG repeat size between transgenic parent and offspring for the various transmissions. The *x*-axis shows CTG repeat length change and the *y*-axis shows the percentage corresponding to each size change for all transmissions in each line. The frequency (%) and mean size (m) of expansions are indicated on the right. The frequency (%) and mean size (m) of contractions are indicated on the left. The number of transmissions assayed for each genotype was as follows: DM300–328, 299; *Msh2* +/– to +/–, 82; *Msh2* –/– to –/–, 82.

Fig. 2. CTG repeat size variations following transgene transmission between parent and offspring carrying different *Msh2* genotypes. The upper genotype indicates parental *Msh2* status and the lower genotype indicates that of the offspring. + is the wild-type allele and – is the mutant allele. In each column, the left bar corresponds to male transmissions and the right bar to female transmissions. The frequencies of expansions (Exp.) and contractions (Cont.) are indicated for each transmission. *n* is the number of transmissions analyzed.

than those obtained for the other DNA repair genes (Table I). In the absence of a functional *Msh2* in both the transgenic parent and the offspring, there was a bias towards contractions (90.2%) rather than expansions of the CTG repeat over generations, for both male and female transmissions. The overall mutability (expansions + contractions) was unchanged in *Msh2*-deficient background, 95.1% in *Msh2* –/– to –/– versus 97.5% in +/– to +/– and 95% in +/+ to +/+ DM300–328 mice, but the direction of instability (towards expansions or contractions) was reversed (Figure 1). Not only was the frequency of mutation similar with and without Msh2, but also the range of size variations, with +14.5 CTG in DM300–328, +12.8 CTG in *Msh2* +/– to +/– and –14.5 CTG in *Msh2* –/– to –/– transmissions. Although *Msh2* knockout mice had a different genetic background compared with DM300–328 mice, *Msh2* +/– to +/– transmissions displayed mostly expansions (78%, +12.8 CTG). A single *Msh2* allele is therefore sufficient to drive instability towards expansions.

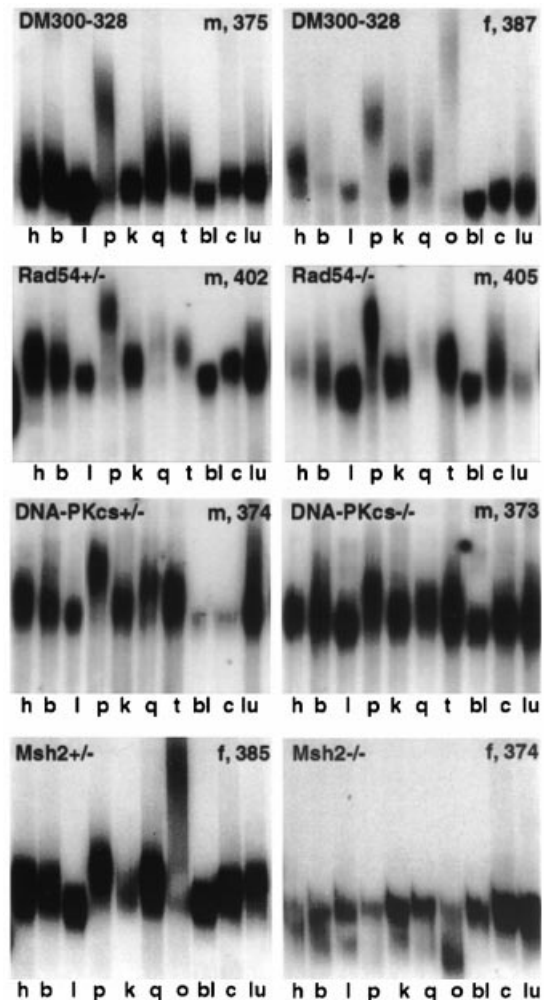### Intergenerational contractions and expansions occur not only in the germline, but also after fertilization

Intergenerational changes in CTG size were analyzed for all possible parental and offspring *Msh2* genotypes, to

elucidate the role of Msh2 in instability (Figure 2). When the transmitting parent carried one functional *Msh2* allele, we observed a bias towards expansions (>78%) in *Msh2* +/+ and +/– offspring, for both males and females. However, when these same *Msh2* +/– transgenic parents gave rise to *Msh2* –/– offspring, we observed mostly contractions (82.2%). These *Msh2* +/– to –/– transmissions had similar instability characteristics to the *Msh2* –/– to –/– transmissions presented above. Both the frequency of contractions (82.2 and 90.2%) and the mean size of these contractions (–13.75 and –14.5) were very similar.

To obtain further insight into intergenerational instability mechanisms, we used single-cell PCR to investigate the distribution of alleles carrying different CTG repeat sizes in individual spermatozoa from 10-month-old males with different Msh2 genotypes. Figure 3 shows CTG repeat sizes in spermatozoa from DM300–328, *Msh2* +/– and *Msh2* –/– males. The length of the CTG repeat sequence in DNA from the tail of the analyzed male was taken as a reference for expansions or contractions, as this measure was used to calculate intergenerational size changes. Furthermore, the tail CTG repeat showed no mosaicism at weaning (3 weeks). A large majority of expansions was obtained in spermatozoa from the DM300–328 (*Msh2* +/+) male (92%, +38.1 CTG) and from the *Msh2* +/– male (91.4%, +17.7 CTG). Expansions were smaller on average in *Msh2* +/– than in DM300–328 mice, probably due to differences in genetic background, or to a decrease in Msh2 expression. For the *Msh2* –/– male, the CTG repeat mutability in spermatozoa was similar to that in the controls, but the size variations were contractions in almost all cases (97.8%, –33.7 CTG). The possible influence of the inherited CTG repeat size on instability dynamics was abolished by choosing mice carrying CTG repeats identical in length. Single-cell PCR profiles for
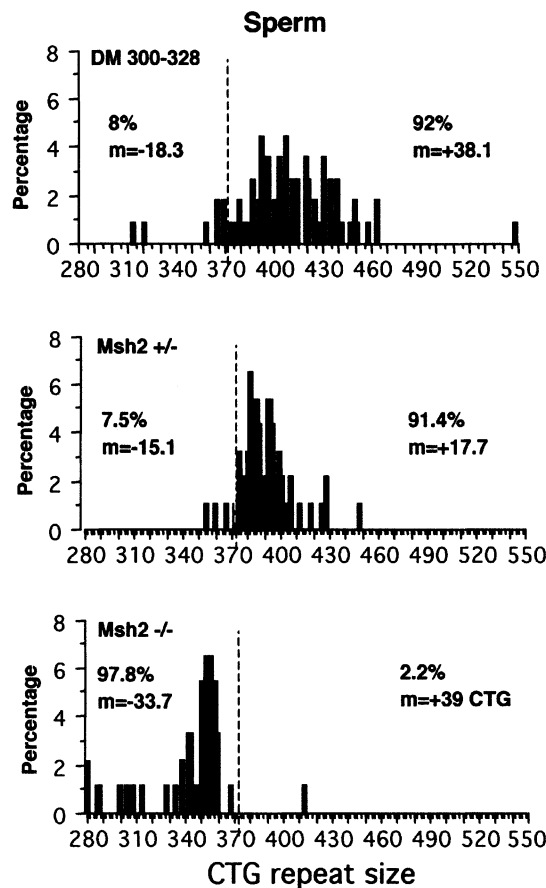
Fig. 3. Distribution of alleles with different CTG repeat sizes in spermatozoa from 10-month-old male transgenic DM300–328, *Msh2* +/– or *Msh2* –/– mice, as determined by single-cell PCR. The *y* axis shows the percentage of each size allele with respect to all single genomes analyzed. The dotted line indicates the CTG repeat size in tail DNA at weaning: 372 CTG for DM300–328, 373 CTG for *Msh2* +/– and 372 CTG for *Msh2* –/–. The frequency and mean size of expansions are indicated on the right and the frequency and mean size of contractions are indicated on the left. The number of single genomes analyzed for each tissue was as follows: DM300–328, 113; *Msh2* +/–, 93; *Msh2* –/–, 92.

Fig. 4. Somatic instability in 10-month-old male and female DM300–328, *Rad54* +/–, *Rad54* –/–, *DNA-PKcs* +/–, *DNA-PKcs* –/–, *Msh2* +/– and *Msh2* –/– transgenic mice. The same instability profiles were observed between *Rad52* +/– and *Rad52* –/– transgenic mice (data not shown). h, heart; b, brain; l, liver; p, pancreas; k, kidney; q, quadriceps; o, ovary; t, testis; bl, blood; c, cerebellum; lu, lung. CTG repeat length in blood is indicated for each mouse. m, male; f, female.

spermatozoa from the DM300–328 male and *Msh2* –/– male were consistent with the percentage of expansions and contractions observed in the respective +/+ and –/– offspring. Previously, it was thought that intergenerational instability results mainly from parental germinal instability. This would result in a strong bias towards expansions in all *Msh2* +/– transmissions, irrespective of the *Msh2* genotype of the offspring. The distribution of expansions and contractions in the *Msh2* +/– male matches those in its +/– offspring, but not in its –/– offspring. Regardless of the sex of the transmitting parent and the *Msh2* genotype (+/– or –/–) of the non-transgenic parent, there was a strong bias in the Msh2 –/– offspring towards contractions rather than expansions (see Figure 2). These results demonstrate that the offspring *Msh2* genotype affects intergenerational instability and that the contraction event in the *Msh2* –/– offspring of an *Msh2* +/– transgenic parent occurs after fertilization. Interestingly, *Msh2* –/– to +/– transmissions displayed an equilibrium between expansions and contractions, suggesting that the presence of a single *Msh2*

allele in the offspring is not sufficient to counteract entirely the effect of germline contractions.

We investigated the timing of this event by assessing somatic instability in various tissues in 1-day-old pups of different *Msh2* genotypes born from parents of various *Msh2* genotypes. Weak intratissue somatic mosaicism was present in most tissues at this stage, with a tendency towards expansions in *Msh2* +/– newborns with *Msh2* +/– parents, and towards contractions in *Msh2* –/– pups from transgenic *Msh2* +/– or *Msh2* –/– parents (data not shown). However, the predominant size of the CTG repeat was similar in the various tissues and we observed no major intertissue mosaicism, suggesting that the second instability event occurred very early in development, probably at the one-cell stage, just after fertilization.

Influence of the various DNA repair genes on somatic instability of the CTG repeat

We assayed somatic instability in 11 tissues in 10-month-old male and female mice, –/– or +/– for each DNA repair
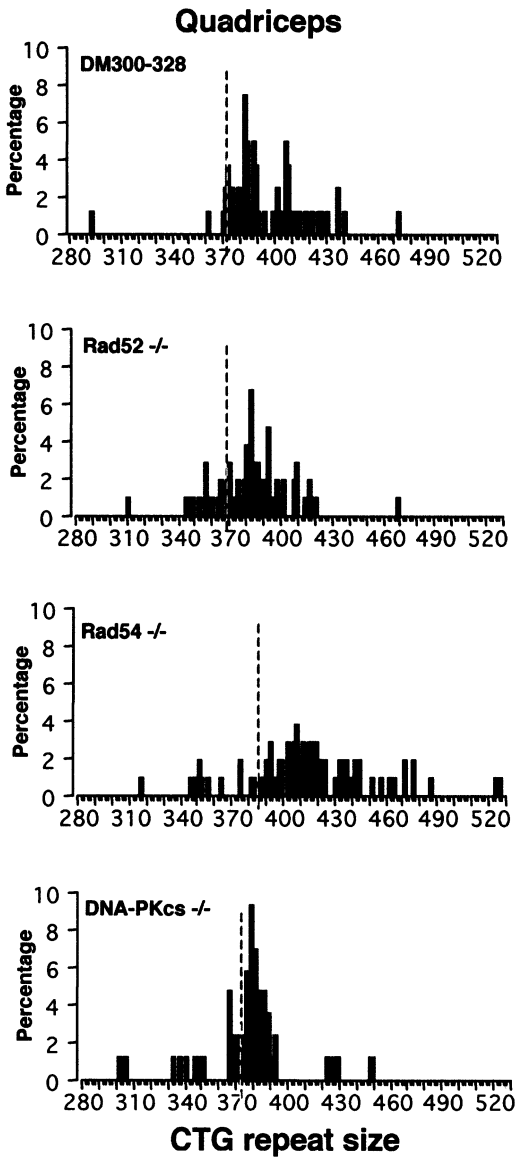
Fig. 5. Distribution of alleles with different CTG repeat sizes in quadriceps from 10-month-old male transgenic DM300–328, *Rad52 –/–*, *Rad54 –/–* and *DNA-PKcs –/–* mice, as determined by single-cell PCR. The dotted line is the CTG repeat size in blood: 372 CTG for DM300–328, 369 CTG for *Rad52 –/–*, 387 CTG for *Rad54 –/–* and 373 CTG for *DNA-PKcs –/–*. The number of single molecules analyzed for each male was as follows: DM300–328: 87; *Rad52 –/–*, 105; *Rad54 –/–*, 106; *DNA-PKcs –/–*, 86.

gene and/or +/+ control mice (Figure 4). We analyzed transgenic mice carrying CTG repeats of identical size in their tail DNA to avoid effects of basal repeat length on instability. Single-cell PCR was also used to resolve the smears and to determine the distribution of alleles with different CTG repeat sizes in several tissues (Figures 5 and 6). CTG repeat length in blood was considered to represent the inherited size in each mouse, because this tissue remains stable throughout the animal's life.

Male and female *Rad52 +/–*, *Rad52 –/–*, *Rad54 +/–* and *Rad54 –/–* transgenic mice displayed similar patterns of somatic instability to control +/+ mice, with a strong bias towards expansions in tissues presenting mosaicism (Figure 4). We also observed mostly expansions when assessing somatic mosaicism in *DNA-PKcs +/–* and –/–

mice, with no differences between mice that did and did not carry a functional *DNA-PKcs* allele (Figure 4). The absence of functional *Rad52*, *Rad54* or *DNA-PKcs* genes in our transgenic mice had no effect on somatic instability, indicating that these genes do not play a critical role in this process. Single-cell PCR performed with DNA from quadriceps, an affected tissue that displays relatively high mosaicism both in DM1 patients and transgenic mice, showed that there was a strong bias towards expansions in DM300–328, *Rad52 –/–*, *Rad54 –/–* and *DNA-PKcs –/–* mice (Figure 5). The mean size of expansions was greatest in *Rad54 –/–* quadriceps, probably due to the larger basal CTG repeat size in this mouse. However, no major differences were observed in the mean size of expansions between DM300–328 and *Rad52 –/–* mice, in contrast to that observed in intergenerational analyses. The lower level of mosaicism observed in *DNA-PKcs –/–* quadriceps may result from the different genetic background carried by *DNA-PKcs* knockout mice.

Somatic instability dynamics were strongly modified in *Msh2*-deficient mice (both in female and male, Figure 4 and data not shown). In control and *Msh2 +/–* mice, a high level of tissue-specific mosaicism was observed, with a strong bias towards expansions, especially in the ovaries and pancreas. In contrast, in *Msh2*-deficient transgenic mice, mosaicism was observed but there was a shift towards contractions.

The results of single-cell PCR studies performed in 10-month-old male mice, with quadriceps (displaying mosaicism in control mice) and cerebellum (displaying little or no mosaicism in control mice) are shown in Figure 6 for *Msh2 +/–*, *Msh2 –/–* and DM300–328 (*Msh2 +/+*) males. In the DM300–328 male, we observed mostly expansions in the quadriceps, with weaker mosaicism in the cerebellum. The *Msh2 +/–* male also displayed mosaicism in both tissues, with a strong bias towards expansions, as observed for females (Figure 4). However, lower levels of mosaicism were observed in the quadriceps of *Msh2 +/–* than in DM300–328, probably due to lower levels of *Msh2* expression or differences in genetic background between DM300–328 and *Msh2* knockout mice. Interestingly, *Msh2 +/–* mice displayed higher levels of instability in the cerebellum than did DM300–328 mice. In sharp contrast, in the *Msh2 –/–* mice, we observed a strong bias towards contractions, with greater size variation in the quadriceps than in the cerebellum. These results show that Msh2 is also necessary to drive instability towards expansions in tissues, and that instability shifts towards contractions in the absence of this gene.

Discussion

To identify the type of DNA repair pathway involved in triplet repeat instability mechanisms, we determined the influence of various DNA repair genes on CTG instability in our transgenic mouse model by monitoring intergenerational and somatic instability in knockout mice for these genes. As CTG repeats have been shown to induce length-dependent breakage in yeast (Freudenreich *et al.*, 1998), we investigated the role of two genes involved in DSBR-HR, *Rad52* and *Rad54*. The absence of *Rad52* or *Rad54* in our transgenic mice had no major effect on intergenerational and somatic instability. The mutability was high
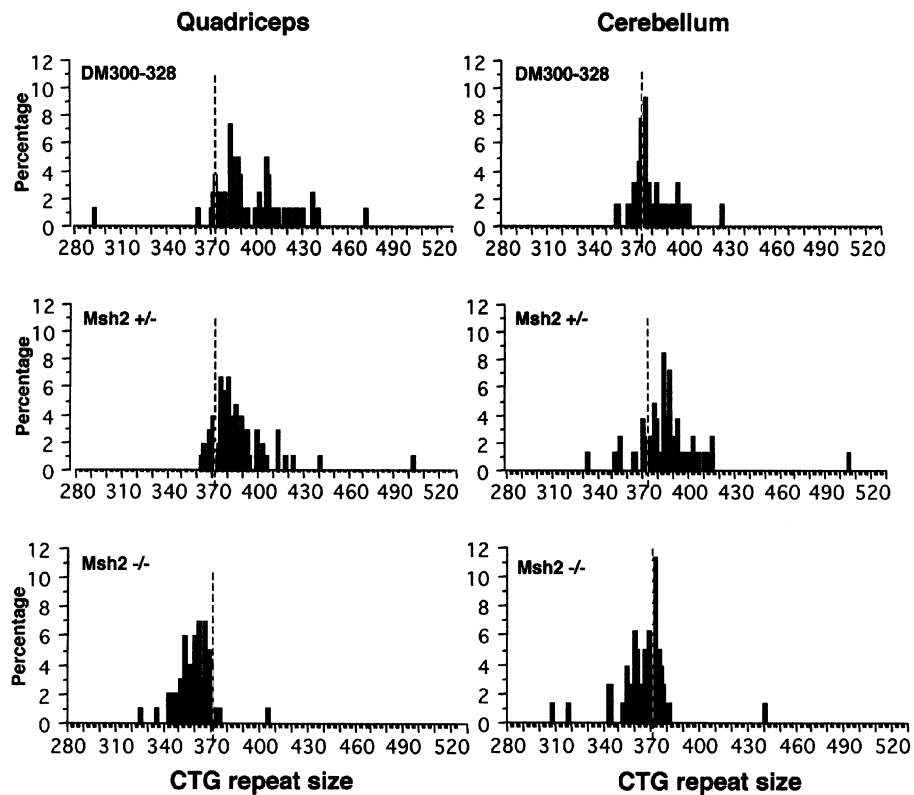
Fig. 6. Distribution of alleles with different CTG repeat sizes in quadriceps and cerebellum from 10-month-old transgenic male DM300–328, *Msh2* +/– and *Msh2* –/– mice, as determined by single-cell PCR. Dotted lines represent the CTG repeat size measured in blood: 372 CTG for DM300–328, 373 CTG for *Msh2* +/– and 372 CTG for *Msh2* –/–. The number of single molecules analyzed for each tissue was as follows, DM300–328: quadriceps, 87; cerebellum, 80; *Msh2* +/–: quadriceps, 106; cerebellum, 83; *Msh2* –/–: quadriceps, 101; cerebellum, 88.

(>90%), and a strong bias towards expansions was observed from generation to generation, in all the tissues tested. Thus, these genes are probably not directly responsible for the CTG repeat instability in our mice and Rad52/Rad54-mediated HR is probably not involved. However, a slight but significant decrease in the mean size of expansions was observed in *Rad52* –/– to –/– transmissions (but not in tissues). Our results with *Rad54* knockout mice suggest that instability is unlikely to be produced through the DSBR-HR mechanism. Thus, if Rad52 is involved in CTG instability, this is probably not via this pathway. Rad52 could possibly act through its activity in single-strand annealing (SSA), which is an error-prone DSB repair pathway that does not depend on other Rad52 group proteins (Paques and Haber, 1999). Interestingly, in yeast, Msh2 has been shown to participate in SSA as well (Pastink and Lohman, 1999). DSBs arising in CTG tracts, due to the formation of aberrant secondary DNA structures during replication, could also be repaired by the NHEJ. The results obtained with *DNA-PKcs* knockout mice showed that instability, and expansions in particular, continue to occur in the absence of DNA-PKcs expression, suggesting that the NHEJ may not affect CTG repeats, at least as far as the action of DNA-PKcs is concerned.

Contrasting results were obtained for mice deficient in *Msh2*. In an *Msh2*-deficient background, high frequencies of mutation were observed in transgenic mice but, in sharp contrast to what was observed in *Msh2*-expressing mice, we observed a large majority of contractions in transgenes

transmitted between parent and offspring and in tissues. These data show that Msh2 is a key factor driving the instability of the CTG repeat towards expansions from generation to generation and in tissues. Msh2 was reported previously to be involved in the generation of somatic expansions in CAG repeats in transgenic mice carrying the first exon of the huntingtin (HD) gene (with ~130 CAG), and the CAG tract was found to be stable in *Msh2*-deficient mouse tissues (Manley *et al.*, 1999). Surprisingly, in our *Msh2* –/– transgenic mice, the CTG repeat was not stabilized but there was a shift in instability from expansions towards contractions in tissues, and during transgene transmissions over several generations. The sequences surrounding the trinucleotide repeat affect the mutability of the repeat, and comparison of the various mice models with trinucleotide repeats suggests that flanking sequences also determine whether instability tends towards expansions or contractions (Gourdon *et al.*, 1997a; Brock *et al.*, 1999; Seznec *et al.*, 2000; Cleary *et al.*, 2002). The presence of large GC-rich human flanking sequences around the CTG repeat, recreating the human genomic DNA environment in our mice, may account for the differences observed in the absence of Msh2 in the different mice models. The majority of contractions observed in transgenic *Msh2*-deficient mice may result from another mechanism, substituting for the action of Msh2 and generating deletions when processing a CTG repeat.

Until now, despite the suggestion of a post-zygotic instability event observed in fragile X-affected fetuses

(Malter *et al.*, 1997), it has been thought that intergenerational instability is mainly the result of germinal instability from the transmitting parent. This germinal instability could take place during stem cell divisions, or at diploid or haploid stages of gametogenesis. It has been suggested that in transgenic mice carrying CAG repeats, expansions occur in spermatozoa by a mechanism involving Msh2 and gap repair (Kovtun and McMurray, 2001). However, in our transgenic DM300–328 mice we observed, using cell sorting and single-cell PCR, that CTG repeat expansion occurs already in spermatogonia (C.Savouret, C.Garcia-Cordier, C.Junien and G.Gourdon, in preparation). We also did not observe any effect of the offspring gender on the CTG repeat intergenerational instability in the DM300–328 mice as has been described in the CAG repeat mouse model (Kovtun *et al.*, 2000). This discrepancy in the behavior of the repeats between these two models is probably related to the different genomic context of the repeats. However, in our transgenic mice, taking into account that the *Msh2* genotype of the offspring is critical in driving the intergenerational instability towards expansions or contraction, instability in the germline is not sufficient to explain changes in CTG repeat size from generation to generation. Detailed analysis of CTG repeat size variation between transgenic parents and offspring carrying different *Msh2* genotypes demonstrated that another event must take place, in addition to gonadal CTG instability. Analysis of somatic CTG instability in tissues from 1-day-old pups carrying various *Msh2* genotypes revealed weak intratissue mosaicism, but no strong intertissue mosaicism, indicating that the second instability event must take place very early during development, probably just after fertilization as suggested for CGG repeat in Fragile X syndrome (Malter *et al.*, 1997). The frequencies of expansions and contractions were similar in *Msh2* –/– to +/– transmissions, showing that contractions in *Msh2* –/– germinal cells were only partially rescued by the presence of a functional Msh2 allele in the offspring, giving rise to both expansions and contractions. Logically, if the effects of the two events were additional, then the mean size of contractions in *Msh2* –/– to –/– transmissions would be expected to be higher than that in *Msh2* +/– to –/– transmissions. However, the frequency and observed mean size of contractions appear to be almost equal for both these transmissions. Factors such as maternal *Msh2* genotype may be critical. If Msh2 is produced during ovogenesis, then a residual fraction of maternal Msh2 proteins produced by a diploid *Msh2* +/– ovocyte may be present in the *Msh2*– haploid ovocyte used for fertilization, potentially modifying CTG repeat instability just after fertilization. This influence of the maternal *Msh2* genotype on stability will be monitored by analyzing a larger number of transmissions in our mice.

The data presented here clearly implicate Msh2 not only in the somatic but also in intergenerational instability of the CTG repeat. As reported previously in another study, Msh3 is also involved in CTG repeat somatic instability which disappears on a Msh3-deficient background (van den Broek *et al.*, 2002). Msh3 probably acts in association with Msh2 forming a Msh2–Msh3 complex, involved in MMR but also in other repair pathways. The competition between Msh3 and Msh6 to form complexes with Msh2

could explain why in Msh6-deficient mice, in which only Msh2–Msh3 complexes are formed, there is an increase of somatic instability (van den Broek *et al.*, 2002). This would imply that only the Msh2–Msh3 complex is involved to generate CTG expansions. The implication of Msh3 in intergenerational instability remains to be demonstrated. However, the mechanism by which Msh2 and Msh3 cause expansions is still unknown, particularly since the Msh proteins may also be involved in other types of DNA repair such as DSBR-HR or SSA. Rad52 may also be involved in the mechanism generating expansions, but probably via a Rad54-independent pathway. Taking these results together, we propose that the Msh2–Msh3 complex generates somatic triplet repeat expansions while repairing DSBs through the SSA pathway. This complex would also generate intergenerational expansions via the SSA, in association with Rad52. Others factors involved in SSA need to be assayed to confirm this hypothesis. In the absence of Msh2, other repair mechanism(s) that remain(s) to be identified, could process the CTG repeat generating contractions. As far as the timing of intergenerational instability is concerned, we suggest that Msh2 and the associated mechanism exert their expansion-producing effects not only in the parental germline, but also just after fertilization, and that the combination of these two distinct events accounts for variations in CTG repeat size over generations. Identification of the precise time at which instability occurs in these two steps should help us to determine the molecular mechanism involved.

Materials and methods

Transgenic mice

Transgenic status was determined by PCR with the DMHR4 and DMHR5 primers. Tail DNA was amplified by PCR, using primers 101 and 102, and repeat length was measured by running CTG amplification products on a 4% acrylamide denaturing gel (Seznec *et al.*, 2000). *Rad52* +/– (C57BL/6 background), *Rad54* +/– (C57BL/6 background) and *Msh2* +/– (129/OLA, FVB background) mutant mice have been described elsewhere (de Wind *et al.*, 1995; Essers *et al.*, 1997; Rijkers *et al.*, 1998). *DNA-PKcs* mutant SCID mice (CB17 background) were purchased from IFFACREDO (France). *Rad52, Rad54, Msh2* and *DNA-PKcs* genotypes were assayed by multiplex PCR, using primers that anneal to either the wild-type or the mutant allele, as reported previously (de Wind *et al.*, 1995; Blunt *et al.*, 1996; Essers *et al.*, 1997; Rijkers *et al.*, 1998).

Somatic instability analyses

Ten-month-old mice were killed and dissected, and DNA was phenol-extracted from tissues (Lia *et al.*, 1998). Semen was taken from the vas deferens and DNA from spermatozoa was extracted with care to ensure that it was not contaminated by DNA from other cells (Seznec *et al.*, 2000). This extraction step is very important, as semen may contain seminal leukocytes and epithelial cells from the epididymis that display a high level of instability, potentially complicating the interpretation of the results. We carried out PCR with 15 ng of DNA from each tissue (DNA from ~2500 cells in total), with 1.4 µM of primers 101 and 102 surrounding the CTG repeat, in a buffer described elsewhere (Jeffreys *et al.*, 1994; Seznec *et al.*, 2000) with 1 unit of Thermoperfect DNA Polymerase (Integro BV, The Netherlands). After initial denaturation for 5 min at 96°C, we carried out 30 cycles of 45 s at 96°C, 30 s at 68°C and 3 min at 72°C followed by a chase of 1 min at 68°C and 10 min at 72°C. PCR products were mixed with formamide loading buffer, heated for 5 min at 100°C and subjected to electrophoresis in 3.5% acrylamide denaturing gels at 55 W for 30 min and 25 W for 13 h 30 min in 1× TBE buffer (0.09 M Tris, 0.09 M boric acid and 2 mM EDTA). DNA was transferred onto a nylon membrane and hybridized with a $(CAG)_{17}$ radiolabeled probe.

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