

Maternal germline-specific effect of DNA ligase I on CTG/CAG instability

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The instability of (CTG)•(CAG) repeats can cause >15 diseases including myotonic dystrophy, DM1. Instability can arise during DNA replication, repair or recombination, where sealing of nicks by DNA ligase I (LIGI) is a final step. The role of LIGI in CTG/CAG instability was determined using *in vitro* and *in vivo* approaches. Cell extracts from a human (46BR) harbouring a deficient *LIGI* (~3% normal activity) were used to replicate CTG/CAG repeats; and DM1 mice with >300 CTG repeats were crossed with mice harbouring the 46BR *LigI*. In mice, the defective *LigI* reduced the frequency of CTG expansions and increased CTG contraction frequencies on female transmissions. Neither male transmissions nor somatic CTG instability was affected by the 46BR *LigI* - indicating a post-female germline segregation event. Replication-mediated instability was affected by the 46BR *LIGI* in a manner that depended upon the location of Okazaki fragment initiation relative to the repeat tract; on certain templates, the expansion bias was unaltered by the mutant *LIGI*, similar to paternal transmissions and somatic tissues; however, a replication fork-shift reduced expansions and increased contractions, similar to maternal transmissions. The presence of contractions in oocytes suggests that the DM1 replication profile specific to pre-meiotic oogenesis replication of maternal alleles is distinct from that occurring in other tissues and, when mediated by the mutant *LigI*, is predisposed to CTG contractions. Thus, unlike other DNA metabolizing enzymes studied to date, *LigI* has a highly specific role in CTG repeat maintenance in the maternal germline, involved in mediating CTG expansions and in the avoidance of maternal CTG contractions.

INTRODUCTION

The expansion of a (CTG)•(CAG) trinucleotide repeat is the genetic basis for at least 15 neurological, neurodegenerative or neuromuscular diseases, including myotonic dystrophy type 1 (DM1) and Huntington's disease (HD) (1). In the normal population, the length of the repeat tracts is genetically stable, ranging from 5–24 repeat units. Tract lengths of >25 repeats can be genetically unstable, while disease-associated expansions of 40–6550 repeats show greatly increased

instability. These mutations are termed 'dynamic mutations' where the repeats continue to expand within the tissues and across the generations. Instability in DM1 and other trinucleotide repeat diseases is thought to contribute to disease pathogenesis in a tissue-specific manner.

Many of the repeat-associated diseases display a parent-of-origin effect where an increased proportion of disease-causing transmissions to offspring arise from either paternal or maternal transmissions of the mutant allele. This parent-of-origin effect is molecularly explained by a paternal

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or maternal repeat expansion bias in the germline, and in some diseases this is coincident with a maternal or paternal repeat contraction bias, respectively. For instance, the strong paternal repeat expansion bias characteristic of most CAG (polyGlu) diseases occurs in spinocerebellar ataxia 1 (SCA1) which also displays a maternal contraction bias (2,3). A strong maternal expansion bias is evident for the very large expansions arising in fragile X mental retardation type A (FRAXA) and DM1, which for the latter lead to the more severe congenital form of DM1 (1). A strong paternal contraction bias is evident for expanded repeats in patients with SCA8, Friedreich's ataxia and FRAXA. Large CTG contractions in DM1 do arise, but these are rare (4,5). Curiously, in maternal, but not paternal transmissions of HD, repeat-length changes were also dependent upon the sex of the offspring, with more expansions and contractions arising in male and female offspring, respectively (6). Equally surprising is the predominant CAG expansions and contractions in male and female progeny, respectively, from the same father HD exon 1 mice (7,8). The absence of CAG tract length differences between X- and Y-bearing sperm suggests that in this HD mouse model the embryo gender-mutation bias occurs postzygotically (7). Little is mechanistically known of either the parental or embryo gender mutation biases or the causes of expansion versus contraction biases.

A role for DNA replication, repair or recombination has been proposed to contribute to repeat expansion (1,9). DNA ligation by DNA ligase is a critical final step common to the processes of DNA replication, repair and recombination. DNA ligase I (LIG1) has been demonstrated to act at replication forks, in long-patch base excision repair (BER), and can act in reconstituted mismatch repair and double-strand break repair (10). LIG1 can affect mutations, for example the addition of human DNA LIG1 to a reconstituted V(D)J recombination system resulted in greater sequence diversity in the coding flanks, particularly in the alterations of direct repeat copy numbers—revealing a potential mutagenic role of this protein (11). *LIG1*-deficiencies lead to errors of fork progression (incomplete processing of Okazaki fragments), long-lived nicks at sites of strand-breaks (12) and widened excision gap sizes during BER. A potential role of LIG1 on CTG repeat instability was suggested in yeast and primate model systems (13–15). These *in vitro* data revealed that the increased levels of functional wild-type LIG1 led to increased instability and suggested that levels of this protein may be a determinant of *in vivo* instability. However, no mammalian *in vivo* system has ever been tested for the role of LIG1 in disease-associated trinucleotide instability.

Mutations in the human *LIG1* gene have been identified in a patient with growth retardation, immunodeficiency and photosensitivity (16). The cell line 46BR.1G1 derived from the patient is homozygous (or hemizygous) for a mutant (Arg-771 → Trp) *LIG1* gene encoding an enzyme with residual ligase activity; it has only 3–5% of the wild-type enzyme activity. In addition to the >20-fold decrease in ligation activity, 46BR.1G1 cells and cell extracts exhibited retarded joining of Okazaki fragments during DNA replication, hypersensitivity to a variety of DNA-damaging agents and delayed ligation, excessive excision and extended BER patch sizes (17–19). Mice engineered to contain the point mutation analogous to that found in human 46BR.1G1 cells

were fecund, showed slow growth, cells with DNA replication failure, genome instability and cancer susceptibility—all phenotypes that are consistent with the DNA ligation defect delaying DNA replication and unligated single-strand DNA breaks, as occur in the human 46BR cells (20). *Lig1* mutant 46BR mice were therefore suitable to assess whether DNA ligation could be involved in CTG repeat instability through parental transmissions and/or in somatic tissues.

In the present study, we have assessed the role of LIG1 in (CTG)•(CAG) repeat instability. Specifically, we assessed the level of CTG instability in transgenic DM1 mice with >300 CTG repeats which were crossed to mice carrying the homozygous 46BR *Lig1* mutation and *in vitro* replication of CTG DNA templates by protein extracts from the 46BR.1G1 *LIG1*-deficient cell line. The 46BR *Lig1* enhanced CTG contractions in maternal transmissions but not paternal transmissions or somatic tissues. The presence of CTG contractions in oocytes and a replication template-specific effect of the 46BR *Lig1* support a role for *Lig1* in maternal allele-specific CTG instability.

RESULTS

CTG/CAG repeats are hyper-sensitive to 46BR LIG1-mediated replication fork progression

To investigate the effect of DNA LIG1 deficiency on DNA replication fork progression through repeat DNAs, we used the SV40 *in vitro* DNA replication system, a model of the primate replication fork. DNA templates containing the SV40 replication origin and 79 (CTG)•(CAG) repeats were replicated using extracts from HeLa cells, containing only wild-type *LIG1*; 46BR.1G1 cells, expressing only the defective *LIG1*; or from the 46BR.1G1 complemented cells that also stably express a transfected wild-type *LIG1* sequence (21) (Fig. 1). The efficiency of complete replication was severely hampered by the *LIG1*-defective 46BR extracts, compared with either the *LIG1*-proficient HeLa or 46BR-complemented extracts (Fig. 1, compare lanes 2 and 6 with 4, lanes 8 and 12 with 10 and lanes 14 and 18 with 16). Strikingly, the proportion of incompletely replicated or aberrantly processed Okazaki fragments is increased for all templates replicated by the *LIG1*-deficient 46BR extracts (compare lanes 2 and 6 with 4; lanes 8 and 12 with 10 and lanes 14 and 18 with 16). The amount of incomplete Okazaki fragments was greatest for the repeat-containing templates; this was particularly exacerbated when CTG served as the lagging strand template (pDM79EF), suggesting that this effect was sensitive to replication direction. Overall, repeat tracts were hyper-sensitive to replication defects by the defective *LIG1*, yielding an accumulation of unligated Okazaki fragments and reduced replication efficiencies which confirm other reports and extend those by revealing CTG/CAG tracts as particularly prone to such errors (17–19). The hyper-sensitivity of CTG/CAG repeats to replication fork progression by mutant 46BR *LIG1* extracts suggested that a similar phenomenon might occur *in vivo* and may contribute to repeat instability. To address *in vivo* the role of a deficient *Lig1* on repeat instability, we used a DM1 transgenic mouse (DM300–328), containing ~45 kb of the human DM1 locus, including a large CTG repeat (>300 units) (22,23).

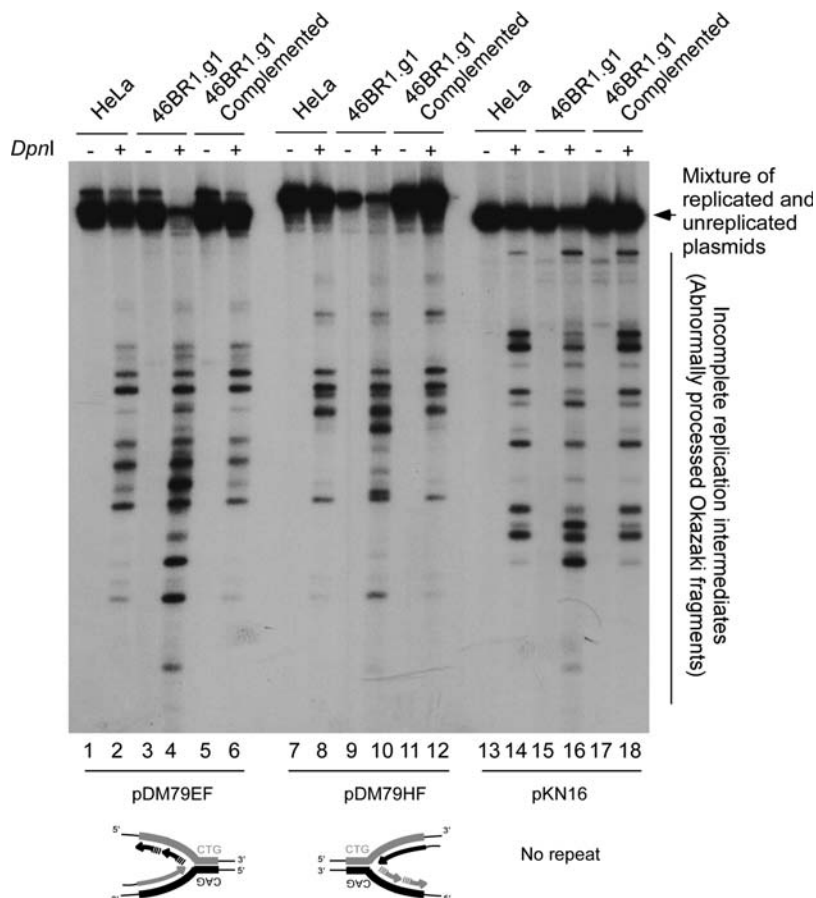


Figure 1. Repeats are hypersensitive to aberrant replication fork progression by 46BR cell extracts. Templates containing SV40-ori only (pKN16) and repeats (pDM79EF and pDM79HF) were replicated *in vitro* by HeLa, 46BR.1G1 and 46BR.1G1 complemented cells. The location of the SV40-ori determine the direction of replication fork progression through the repeats and thus which strand (CTG or CAG) served as a lagging strand template for Okazaki fragment synthesis. Purified products were linearized with *Bam*HI, and some of this material was also digested with *Dpn*I. Products of primate complete replication were separated from incompletely replicated material by *Dpn*I digestion, which cleaves only bacterially derived DNA. The *Dpn*I-resistant material represents the products of at least one complete round of replication or one incomplete round of primate DNA replication (*Dpn*I cleaves the region not replicated but does not cut the region which initiated the replication). The digested products were electrophoresed on a 1% agarose gel to resolve the completely replicated and un-replicated material. An autoradiogram of the dried gel is shown.

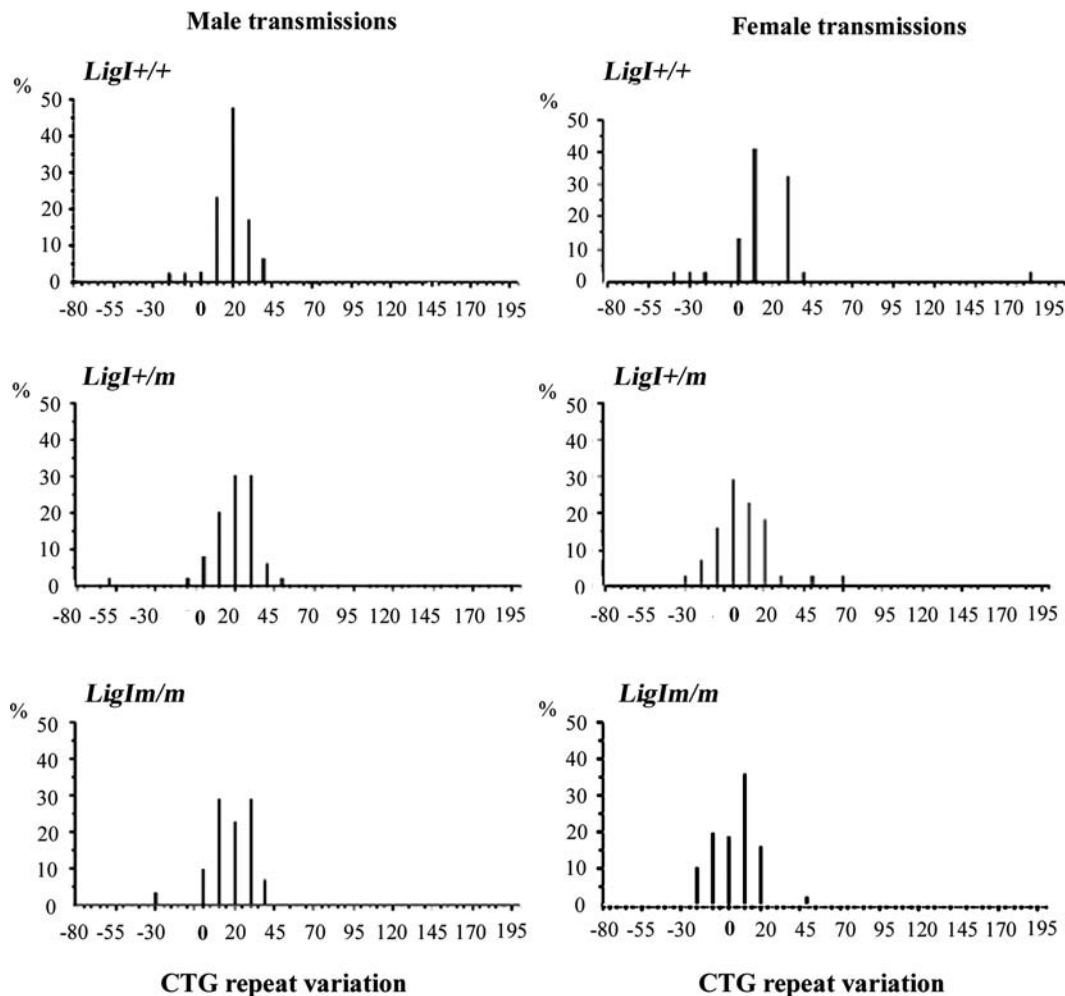
Intergenerational CTG instability in mice carrying the 46BR *LigI* mutation

DM300–328 mice display >90% CTG repeat length variation over generations and high levels of age-dependant somatic instability, both showing a strong bias towards expansions (22,23). 46BR *LigI* mutant mice (20) were crossed with DM300–328 mice by successive breeding over generations. CTG repeat length variations after male and female transmissions were analysed in mice carrying either the wild-type *LigI* gene (*LigI*^{+/+}), 46BR heterozygous (*LigI*^{+/m}) or 46BR homozygous mutant *LigI* gene (*LigI*^{m/m}). Frequencies of intergenerational CTG expansions and contractions were determined by measuring CTG repeat length changes in the tail DNAs of offspring compared with CTG lengths of the transmitting parent (Table 1 and Fig. 2). The frequency of CTG expansions is high in mice also containing the wild-type *LigI* (*LigI*^{+/+} to *LigI*^{+/+} transmissions; 94 and 78% in male and female transmissions, respectively). The magnitude of expansions ranged from 10 to 40 (with one event at 180 CTG for the female transmissions), and contractions ranged

from –10 to –40 CTG. This expansion bias, mutation frequency and magnitude were similar to previous observations (22,23). For the mice with *LigI*-deficient backgrounds (*LigI*^{+/m} and *LigI*^{m/m}), no significant differences were observed in the frequencies or ranges of expansions and contractions for male transmissions (Table 1 and Fig. 2). However, female transmissions showed both a significant decrease in the frequency of CTG expansions and a significant increase in the frequency of contractions when one or both *LigI* alleles were mutated ($P = 0.017$ and $P = 0.028$, respectively). The mutation frequency and the ranges of mutations were similar in the *LigI*^{+/m} to *LigI*^{+/m} and *LigI*^{m/m} to *LigI*^{m/m} female transmissions, suggesting that the heterozygous *LigI* mutation is sufficient to disrupt the level of CTG instability. The frequency of expansions was lower in these two types of transmissions compared with the *LigI* wild-type female transmissions (47 and 53% compared with the 78% expansions observed in *LigI*^{+/+} transmissions, Table 1). The frequency of contractions was higher in the *LigI*^{+/m} to *LigI*^{+/m} and *LigI*^{m/m} to *LigI*^{m/m} female transmissions (24 and 29%

Table 1. Intergenerational CTG repeat instability in transgenic mice carrying the 46BR *LigI* mutation

Parent-to-offspring transmission	Transmissions analysed	Expansion frequency (%)	Contraction frequency (%)	Mean exp. (CTG)	Mean contr. (CTG)
Male transmissions					
<i>LigI</i> ^{+/+} to <i>LigI</i> ^{+/+}	48	94	4	+21	-15
<i>LigI</i> ^{+/m} to <i>LigI</i> ^{+/m}	50	88	4	+23	-35
<i>LigI</i> ^{m/m} to <i>LigI</i> ^{m/m}	31	87	3	+21.4	-30
Female transmissions					
<i>LigI</i> ^{+/+} to <i>LigI</i> ^{+/+}	36	78	8	+25	-30
<i>LigI</i> ^{+/m} to <i>LigI</i> ^{+/m}	45	47	24	+19.5	-14.5
<i>LigI</i> ^{m/m} to <i>LigI</i> ^{m/m}	51	53	29	+14.4	-13.3

**Figure 2.** Intergenerational instability of CTG repeats in wild-type and *LigI* mutant mice. The x-axis shows CTG repeat length changes and the y-axis the percentage corresponding to each size change for all transmissions analysed. Genotypes of parent and offspring are indicated on the upper left of graphs.

compared with the 8% observed in the *LigI*^{+/+} transmissions, Table 1). The age of the transmitting mothers did not affect the contraction events detected either in the *LigI*^{+/m} or *LigI*^{m/m} mice (ages ranged from 9.5 to 28.5 weeks, 45 + 51 maternal transmissions, respectively), since no significant correlation was found between CTG repeat changes and ages. Thus, the 46BR *LigI* mutation affects intergenerational CTG instability in maternal but not paternal transmissions yielding a bias towards contractions.

Somatic instability in mice carrying the 46BR *LigI* mutation

To determine whether the mutant *LigI* affected somatic CTG repeat instability, the length of the CTG tract in the DNA from various tissues was assessed by polymerase chain reaction (PCR) amplification across the repeat tract. Each tissue in a wild-type *LigI*^{+/+} mouse showed a distinct pattern of CTG expansions, as previously observed (23). The tissue-

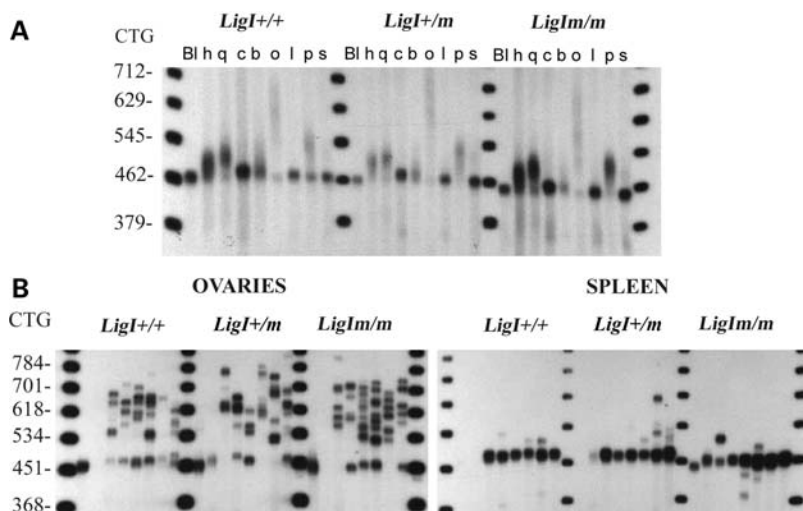


Figure 3. (A) Somatic instability in 19-month-old *LigI*^{+/+}, *LigI*^{+/m} and *LigI*^{m/m} transgenic females. Bl, blood; h, heart; q, quadriceps; c, cerebellum; b, brain; o, ovaries; l, liver; p, pancreas; s, spleen. (B) Representative small pool-PCR (SP-PCR) analyses of CTG repeats in DNA molecules extracted from 19-month-old transgenic mouse tissues. The autoradiographs show representative SP-PCR analyses of DNA extracted from ovaries and spleen. Genotypes are indicated. m: 250 bp DNA ladder converted to number of CTG repeat units in the given PCR product. *LigI*^{+/+}, *LigI*^{+/m} and *LigI*^{m/m} represent genotypes at the murine locus for *LigI*. +: wild-type allele; m: 46BR mutation. The *LigI*^{+/+}, *LigI*^{+/m} and *LigI*^{m/m} females in tail-extracted DNA at weaning contained about 500 CTG repeats for each genotype.

specific, expansion-biased pattern of CTG instability was unaltered by the status of *LigI* (Fig. 3A). Stability was unaffected in the spleen, which was previously demonstrated in the *LigI*^{m/m} mice to display low levels of genetic instability as assessed by the micronuclei assay (20). To confirm the absence of an effect of LIGI activity on somatic CTG instability, we assessed CTG instability by the highly sensitive small-pool PCR which can detect rare CTG-length variations in tissues, such as ovaries and spleen (24). No difference was observed in the levels of the somatic CTG repeat between mice with the wild-type or mutated *LigI* (Fig. 3B, and data not shown).

Overall, the decreased LIGI activity has a specific CTG contraction bias effect through the maternal transmissions, not observed after paternal transmissions or in somatic tissues. The instability exclusively in maternally transmitted CTG alleles may be explained by unique events occurring in the female reproduction system. Such events could include LIGI expression/activity levels in ovaries and/or aberrant processing of DNA structures that form in the repeats during oogenesis or during post-zygotic replication of the maternal allele.

Status of LIGI protein in DM300–328 mice tissues

To assess whether the expression of LIGI protein might explain the preferential sensitivity of the maternal transmissions to both increased CTG contractions and decreased expansions by the mutant *LigI* mice, we assessed the level of LIGI in various tissues of DM300–328 mice with the different *LigI* genotypes (Fig. 4). Maternal and paternal germinal tissues (testis and ovaries) showed considerable levels of LIGI, which did not vary between *LigI* genotypes (Fig. 4A). However, for all genotypes, the amount of LIGI was 20- to 30-fold greater in testis than in the ovaries. This result possibly indicates varying and specific functional requirements of LIGI

in testis compared with ovaries, but does not offer a direct explanation of the instability towards contraction observed in *LigI*^{m/m} female mice. In somatic tissues, the LIGI levels were low or near undetectable with the exception of spleen and ovaries (Fig. 4B). In all cases, the LIGI amounts did not vary between mice genotypes. The phosphorylation status of LIGI was assessed in all tissues, since the level of LIGI phosphorylation can affect repair activity (25–27) and may also affect patterns of CTG instability. The levels of LIGI or its phosphorylation status between tissues did not show an obvious correlation with the tissue-specific patterns for CTG instability; further supporting a limited contribution of LIGI to somatic instability. Importantly, LIGI phosphorylation, such as LIGI levels, did not vary between mice *LigI*^{+/+}, *LigI*^{+/m} and *LigI*^{m/m} for somatic and germinal tissues (Fig. 4B and C, respectively). Thus, the LIGI phosphorylation status is invariable between genotypes for each tissue and is unlikely to be the source of the distinct female intergenerational CTG instability observed in mice expressing mutant *LigI*.

We propose that a combination of a maternal germline-specific function of *LigI* coupled with the reduced activity of the mutant LIGI render the oogonia or the earliest post-zygotic genome duplications of the maternal CTG allele, particularly sensitive to CTG contractions, even with a heterozygous reduction in the level of wild-type *LigI*. It is unclear whether the CTG contraction events arose in the oogonia or during the post-zygotic stages.

CTG contractions are present in oocytes

The effect of the mutant *LigI* upon maternally transmitted CTG instability in DM1 mice may be the result of DNA metabolic processes unique to oogenesis or to the maternal genome following fertilization. Either the CTG contraction events arose during the production of the maternal primordial germ

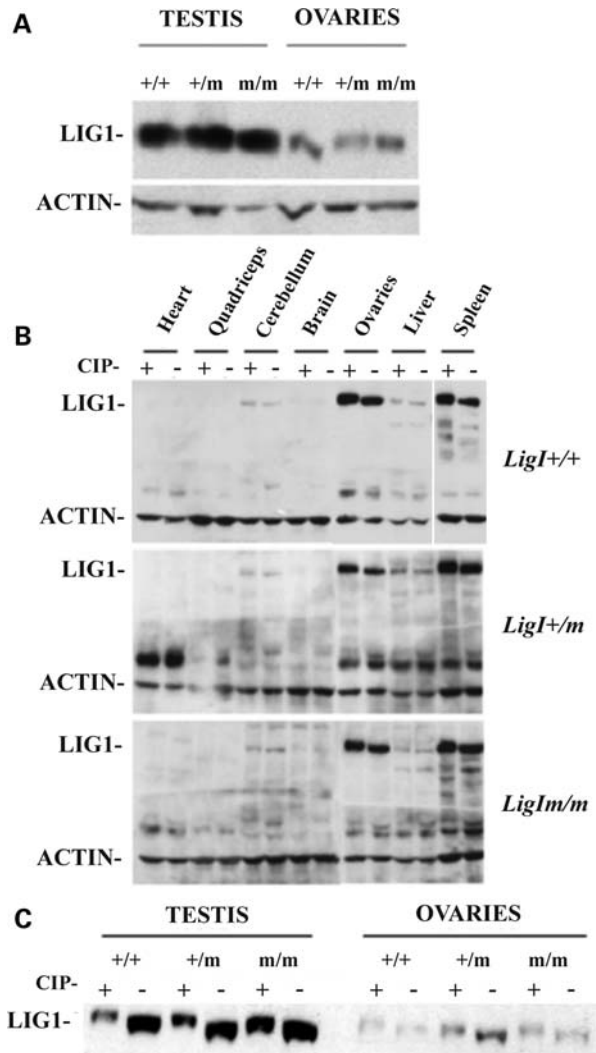


Figure 4. Characterization of LIG1 status in from 3- to 4-month-old mice carrying different *LigI* genotype. (A) LIG1 levels in germinal tissues (B) LIG1 levels and phosphorylation status in several somatic tissues. (C) LIG1 phosphorylation status in germinal tissues. Actin was used as a protein control.

cells, during their long-term arrest in Meiosis I or as a post-fertilization event unique to the maternal CTG alleles. In the former two cases, one would expect the altered pattern of CTG instability to be present in the oocytes of a fertile 46BR mother. If contraction events were limited to the post-fertilization stages, one would not expect oocytes to harbour contractions. Towards addressing whether instability arose during the oogenic process or during post-zygotic stages, we determined whether the maternal contractions from either the *LigI*^{+/+} or *LigI*^{m/m} mother are present in their oocytes. Oocytes were isolated from superovulated fertile mothers of *LigI*-proficient or the *LigI*^{m/m} backgrounds and DNAs isolated from these, their follicular tissues and tails (Fig. 5). Repeat length analysis revealed CTG expansions in the follicular tissue [compared with the CTG repeat in tail DNA at weaning, previously shown to represent the inherited CTG repeat size (28) in both strains]. However, large contractions of repeat lengths relative to the tail were observed in the

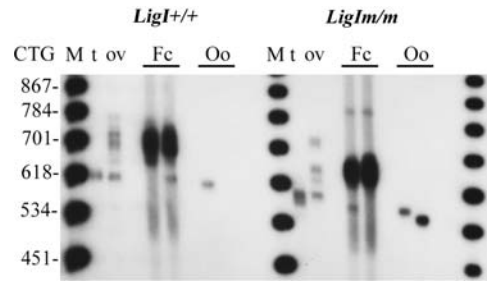


Figure 5. Somatic instability in *LigI*^{+/+} and *LigI*^{m/m} transgenic females. t, tail; ov, ovaries; Fc, follicle cells; Oo, oocytes. m: 250 bp DNA ladder converted to number of CTG repeat units in the given PCR product. +: wild-type allele; m: 46BR mutation.

oocytes of the *LigI*^{m/m} mice. These results support the occurrence of pre-fertilization CTG contraction events in the female germline, which may also continue as maternal allele-specific CTG contractions during post-fertilization stages.

46BR-*LigI* CTG contractions depend upon replication program

The effect of the mutant *LigI* on intergenerational instability observed exclusively in female transmissions may be explained by DNA metabolic processes unique to oogenesis. For example, the DNA replication program of the genome in the primordial germ cell divisions that produce oogonia is distinctly different from somatic and male germinal cells (29,30). These differences are specifically due to variations in the number and location of active replication origins. Similarly, the replication program of the maternal genetic complement in the earliest post-fertilization cell divisions is different from that of the paternal pronuclei (29,30). To test the hypothesis that altering the location of replication initiation relative to the repeat can, in the context of a mutant 46BR *LIGI*, alter CTG instability, we assessed the repeat instability using a defined replication system (31). Repeat instability was assessed following replication of a series of circular DNA templates with a tract of 79 repeats that had the location of the SV40 replication origin placed, by cloning site selection, at varying distances from the repeat (Fig. 6). These DNA templates were replicated *in vitro* using *LIGI*-deficient and -proficient cell extracts and the SV40 replication initiator T-antigen and replication products assessed for instability using the STRIP assay (see Materials and Methods).

A significant *LIGI*-specific effect upon CTG contractions was evident for template pDM79BF (Fig. 6, compare instability following replication by 46BR and 46BR-complemented extracts, $P = 0.03$). This *LIGI*-specific effect depended on the location of the replication origin relative to the repeat, as another template pDM79ER with the SV40-replication origin shifted closer to the CTG repeat tract by ~100 nt, through cloning site selection, did not show a *LIGI*-specific effect upon CTG instability (Fig. 6). The enhanced CTG contractions for pDM79BF by the *LigI*-mutant 46BR extracts were similar to the 46BR mutant *LigI* effect on CTG contractions in maternal transmissions (Table 1 and Fig. 2). These results suggest that the replication profile differences may place the

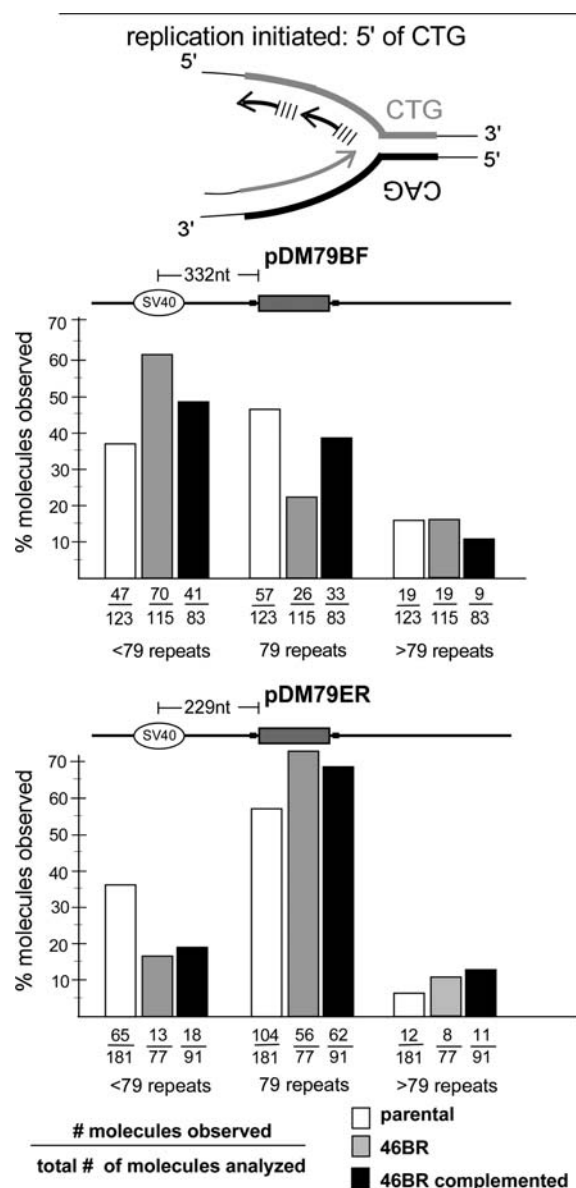


Figure 6. Mutation analysis by measuring the repeat lengths before and after replication by *LigI* deficient (46BR) and *LigI* deficient complemented (46BR complemented) cell extracts. The panel at the top shows the direction of replication and which strand will serve as the leading or lagging strand template. The actual number of molecules scored with different length distribution (>79, 79 or 79<) are indicated over the total number of molecules analysed. Exact numbers are reported. The y-axis represents the percentage of molecules observed. The three panels depict the distribution of repeats before replication (open bars) and after *in vitro* replication by 46BR (grey filled bars) and 46BR complemented (black filled bars) cell extracts for plasmid pDM79BF and pDM79ER. The statistical significance of the results is determined by comparing the frequency of stable, contraction or expansion of one group with another group by using χ^2 test. The value <math>P < 0.05</math> was considered significant. pDM79BF showed increased CTG instability ($P = 0.03$) biased towards contractions ($P = 0.01$) when replicated by 46BR extracts compared with replication by 46BR-complemented extracts ($P = 0.03$).

repeat tract in a contraction-prone location that is particularly sensitive to *LIGI* activity. However, we cannot know which replication origin may be used in oocytes. Our data are consistent with the maternal-specific altered instability occurring

either as a replication event unique to the cell divisions that produce the maternal germ cells or possibly also as a post-fertilization event unique to the maternal CTG alleles during the earliest embryonic cell divisions.

DISCUSSION

We observed no change in the levels of somatic instability between *LigI* mutant mice and wild-type mice. The mutant *LigI* did not affect CTG repeat instability in offspring of paternal transmissions. Nevertheless, a significant decrease in the frequency of CTG expansions coupled with an increase in the frequency of CTG contractions arose when the repeats were transmitted by females carrying the 46BR mutation on one or both alleles. These results suggest that DNA *LIGI* is not required for either somatic expansions or paternal expansion, but is involved in the formation of maternally transmitted CTG expansions and in the avoidance of maternal CTG contractions. These effects are consistent with underlying gender specificities in the processes of CTG instability, as well as a maternal-specific role for *LigI*.

The enhanced CTG contractions upon maternal transmissions were similar under heterozygous and homozygous 46BR genotypes, suggesting that the presence of a single copy of the mutant 46BR *LigI* may have a dominant negative role for some but not all functions, which might include maternal CTG stability. This possibility was previously suggested by (i) the *LigI*-null mouse cells performed better in the survival and replication assays than did the 46BR.1G1 mouse cells, supporting the concept that the mutant form may have deleterious effects over the compensation by other ligase activities (32); (ii) the partial correction ($\pm 60\%$) of cell sensitivity to ethyl methanesulphonate treatment in 46BR.1G1 cells transfected by the wild-type *LIGI* gene (21); (iii) the mutant 46BR *LIGI* increased lifetimes of the mutant protein upon DNA intermediates (17), altered dynamics of protein-protein interactions or alterations of either protein-mediated modifications of *LIGI*, such as phosphorylation (33). While these data might suggest an aberrant dominant-negative property of the mutant ligase, the more likely possibility is that a 50% reduction in normal *LIGI* levels is sufficient to trigger the phenotype.

There was no strong or clear effect of the 46BR *LigI* mutation on somatic CTG instability. Two papers revealed an enhanced CTG instability with over-expression of wild-type *LIGI* in human cells or in yeast (13,15)—which might suggest a possible correlation of *LIGI* levels or activity between tissues displaying variant patterns and levels of CTG instability. The high levels of CTG instability in the heart, quadriceps, brain and pancreas, which show the lowest levels of *LIGI*, compared with an absence of CTG instability in the cerebellum, liver and spleen, which show varying levels of *LIGI*, suggest a lack of correlation of *LIGI* expression levels with instability or that if there is a correlation, it is complex. This conclusion is based only upon inter-tissue *LIGI* expression differences and cannot take into account other *cis*- or *trans*-factors that may vary between tissues. The absence of an obvious effect of the 46BR mutant *LigI* on genomic instability in general (32) is

consistent with an absence of a strong role in somatic CTG instability.

In the absence of MSH2, only 5% of offspring showed expansions relative to their parents (instead of 90%) and ongoing somatic expansions do not occur (34–41). Interestingly, CTG contractions rather than expansions predominated, with 90% expansions versus 90% contractions in the MMR-proficient and MMR-deficient states, respectively. This shift in the mutation direction occurred by paternal or maternal transmissions. However, Foiry *et al.* (41) observed a maternal bias for CTG contractions in transmissions from mothers with heterozygous deficiencies of the *Msh3* gene, suggesting that the maternal transmissions are more sensitive to CTG contractions in the absence of DNA repair proteins than paternal transmissions. In the present study, *LigI* deficiency also led to a shift from expansions to contractions, but this was observed only in maternal transmissions; indicating a unique role of DNA LIGI in maternal genome stability and distinguishing its role from that of MSH2 and MSH3.

We have also observed that the age of the transmitting mothers did not affect the intergenerational CTG instability in either the *LigI*^{+/m} or *LigI*^{m/m} mice. This argues against a strong post-meiotic genome maintenance/repair mechanism for the increased repeat contractions and decreased expansions arising in the *LigI*-deficient mice. In contrast, Kaytor *et al.* (2) have shown a maternal age effect for CAG contraction frequency and magnitude in SCA1 mice. The altered instability might occur as a post-fertilization event, where one would not expect the altered pattern of instability to be present in the oocytes of a fertile mother. However, the timing of the instability event may occur as early as germline segregation (5 days post-fertilization, prior to ovulation) while the unborn mother is still a developing embryo. However, contractions must have arisen following germline segregation since if they arose before this we would have detected contractions in somatic tissues, which we did not. We revealed the presence of CTG contractions in oocytes (meiotic stage) from homozygous 46BR mice, which suggests that the contractions would have occurred prior to meiosis and might have arisen during a proliferative stage of germline development (possibly during DNA replication). Our data also show that the effect of the mutant 46BR *LIGI* upon instability depends upon the replication program. The absence of an altered pattern of instability by the mutant 46BR *LIGI* for the majority of templates, with the exception of pDM79BF, is consistent with the unaltered patterns of paternal intergenerational instability and somatic instability in the various tissues of the ligase-deficient DM300–328 mice (data not shown, Fig. 6). In contrast, the instability is biased towards CTG contractions in the mutant *LIGI* for the pDM79BF templates, which is consistent with maternal instability observed in the ligase-deficient DM1 mice. Factors affecting the location of Okazaki initiation can affect the location of repeats in the replication fork and may determine instability.

We propose that the replication profile unique to the maternal DM1 locus during either the cell divisions leading to the production of the oogonia or the earliest post-zygotic divisions predispose the CTG repeat to contractions in the presence of a mutant *LigI* (Fig. 7). Specifically, the location of the replication origin used to replicate the maternal DM1 repeat during the cell

divisions to produce the oogonia, or the earliest post-zygotic cell divisions, may be placed closer to or further from the repeat relative to later stages of development. Such changes may be mediated by alterations in chromatin packaging, which have recently been reported to occur during those early developmental stages (42). The replication program during these stages coupled with the presence of the mutant 46BR *LigI* could predispose the maternal expanded CTG allele to contractions. In mice, the maternal genome displays a unique replication program during oogenic cell divisions and during early-post-zygotic pronuclei and subsequent cell divisions (29,43–46). Furthermore, we recently observed distinct replication initiation profiles between tissues of DM1 transgenic mice (47). During the mapping of the replication origins within the ~45 kb human DM1 transgene, we observed only one active origin in the region and this was proximally located downstream of the CTG tract. This supports the conclusion that the repeat is most likely replicated by replication forks originating from this origin, rather than from one occurring within the murine genome adjacent to the transgene integration site. We also observed development-specific alterations in the replication profile of the DM1 locus in testes, where a sufficient amount of nascent DNA could be collected and assessed. Similarly, replication profile variations unique to the female germline may also arise, but could not be assessed due to limiting DNA amounts from these few cells. Evidence suggests that the number of replication origins used to replicate mouse oocytes is 3-fold fewer than that used to replicate spermatogonial genomes (29), which is consistent with our proposal that there is a specific replication program unique to oogonal replication (Fig. 7). Such variations may lead to CTG contractions in the presence of the mutant 46BR *LigI*.

This mechanism (Fig. 7) could apply to the replication profile of the maternal DM1 allele during earliest post-zygotic cell divisions. Notably, CTG contractions have been observed in human DM1 embryonic stem cells, suggesting that early post-fertilization mutations may also be active (48). Replication of paternal pronuclei differs from that of maternal pronuclei and from that of zygotic nuclei (49–51). The first round of post-zygotic replication is gonomic (52), such that the paternal and maternal pronuclei undergo full replication prior to fusing nuclear membranes. The replication timing of the paternal genome is ~20% faster than that of the maternal genome, beginning and finishing earlier (43–46). The maternal longer replication timing can be indicative of slower fork progression and/or a replication program with activation of fewer origins. The role of chromatin in mediating specific replication profiles at the DM1 locus is supported by our recent observations in specific tissues of the DM1 mouse (47) and chromatin changes during early development (42).

A maternal-specific effect of mutation predisposition by a heterozygous deficiency of a DNA repair gene, *Pms2*, has previously been reported (53,54). The *Pms2* deficiency of maternal pronuclei and one to eight cell embryos derived from *Pms2*-deficient oocytes persisted and increased the susceptibility of the mice to non-trinucleotide microsatellite instability. These groups proposed that the inherited mutations arose during the earliest rounds of embryonic cell divisions. While they had not tested the mutation level in oocytes to assess the possible effect of primordial germ cell divisions

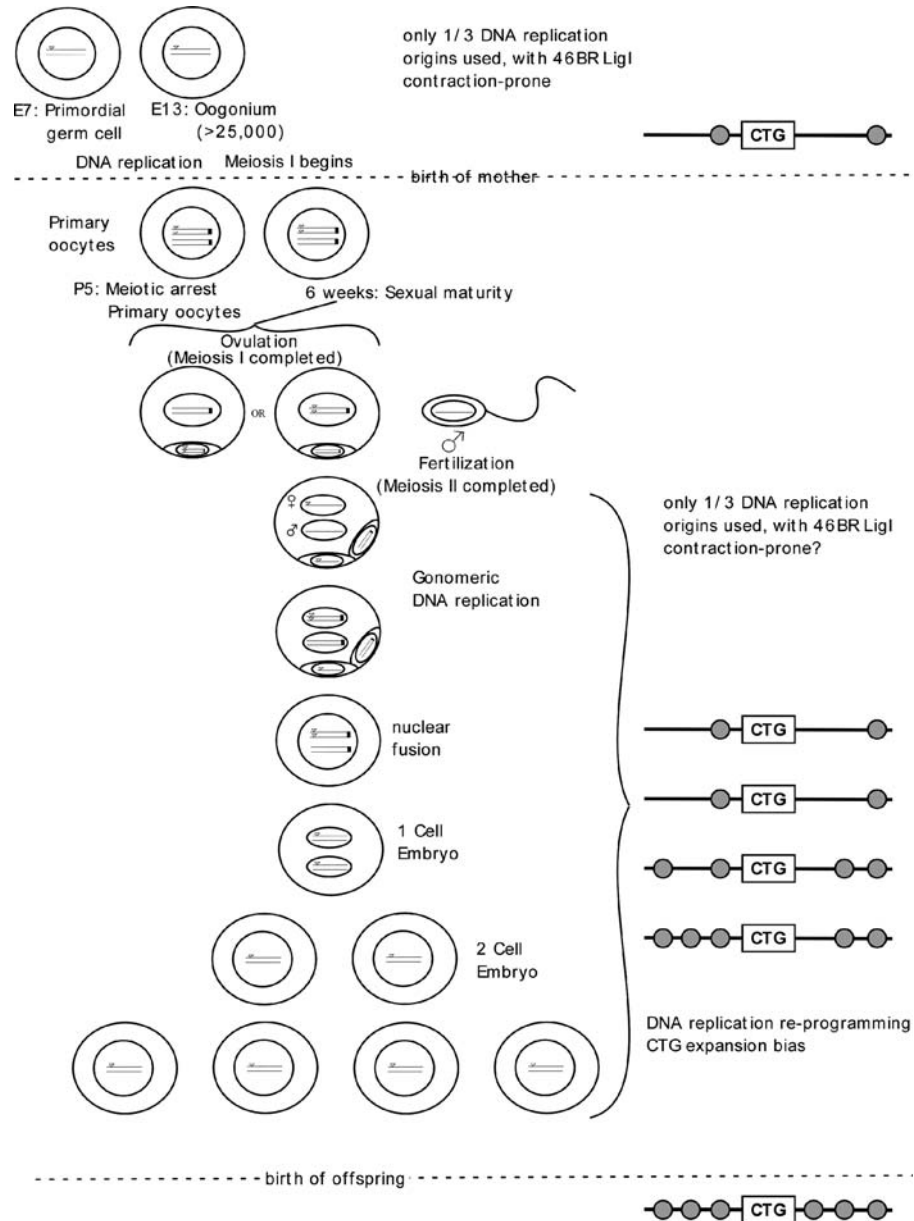


Figure 7. Oocyte development, post-zygotic divisions and CTG repeat instability. A population of ~50 primordial germ cells is present at embryonic day 7 (E7). These primordial germ cells divide with active DNA replication, then migrate, colonizing each gonad primordium by embryonic day 13 (E13). These oogonia cease proliferation and enter meiosis, arresting at the diplotene stage of meiotic prophase I, called dictyotene, by postnatal day 5 (P5). Meiosis I is completed just prior to ovulation. Meiosis II is not completed until after fertilization. The first round of DNA replication occurs in two pronuclei, with maternal and paternal genomes separated. Only after this gonameric replication do the genomes mix with nuclear fusion, segregation and subsequent rounds of cell division. The CTG tract is indicated with an open triangle. Sister chromatids are connected with a filled oval. The number of replication origins activated in the maternal genome during female gametogenesis and the earliest rounds of embryonic cell division (shown to the right by dots) is one-third of those activated in the somatic tissues or the paternal genetic complement during fertilization (29,30). In the fertilized egg, the maternal and paternal genomes are separately compartmentalized in male and female pronuclei and each undergoes DNA replication independent of the other and only following this first round of gonameric genome replication do the two haploid genomes mix to become a zygote (52). Furthermore, in one-cell embryos, the male and female pronuclei replicate asynchronously and replications of two cell embryos and blastomeres are synchronous (45,46,49,51). A schematic of the DM1 locus and potential replication origins utilized through development is shown to the right. Coupled with a *Ligt* deficiency, the replication origin activated in the maternal genome during female gametogenesis and the early rounds of embryonic cell division may permit an enhanced formation of CTG contractions. See text for description.

upon mutation status, this may be further exacerbated during the very first round of post-zygotic DNA replication which is gonameric—where the maternal and paternal genomes, physically separated in the maternal and paternal pronuclei, replicate independently, and mix only after the first round of replication. Our findings presented herein are the first

maternal-specific effect for a mutant *Ligt* and for trinucleotide repeats.

In other trinucleotide repeat diseases, a contraction bias of expanded CTG•CAG repeats through maternal but not paternal transmissions has been observed in humans for the SCA1 locus, and in many but not all mouse models harbouring

expanded CTG•CAG repeats. Among these are SCA1 cDNA CAG82 transgenic mice (2), Sca178Q/Sca12Q (55), Hdh4/Q80 knock-in mice (56), HdhQ18-Q111 knock-in mice (57), huntingtin exon 1 transgenic mice (CAG)113–156 (58), androgen receptor yeast artificial chromosome transgenic mice (CAG45) (59) and DRPLA YAC transgenic mice (CAG76–78) (60). All these mice displayed a bias of small contractions in maternal transmission, an effect not occurring in DM1 transgenic mice with (CTG)55 or (CTG)300 tract lengths (22,23). These results suggest that the mechanism underlying gametic repeat instability is distinct in females and males. It is noteworthy that the female germline mutation frequencies of Sca178Q/Sca12Q and SCA1 cDNA CAG82 D02 mice were similar to each other but considerably higher than those of Hdh4/Q80 and DRPLA YAC transgenic mice (CAG76–78) (56,60), which contrasts with their absence in DM1 mice (22,23).

Thus, CTG contractions in female transmissions depend on unknown *cis*-acting sequences that are present near or adjacent to the repeat, as well as the length of the repeat (55). Our observations with the DM1 *LigI* defective mice suggest that a *cis*-element specific to the maternal gametic genome marks it for CTG contractions. Our data are consistent with this *cis*-element being the replication program of the DM1 locus following germline segregation and possibly through to the earliest embryonic cell divisions.

MATERIAL AND METHODS

SV40 replication templates

The construction of SV40 replication templates is described in Cleary *et al.* (31). Briefly, genomic clones of DM1 containing *EcoRI/HindIII* (CTG)*n* fragments were sub-cloned into *pBluescript KSII+* (Stratagene) (61,62). The *SphI-HindIII* fragment (viral nucleotide positions 128 and 5171, respectively) of the SV40 virus, which contains the SV40-*ori*, was amplified by PCR with primers (5'-CTTATCTCTAGAAGCTTTTT GC-3' and 5'-CGCTCTAGATGGTGCATGCATCTC-3'). The PCR product of 219 bp was inserted into *pBluescript KSII+* using the *XbaI* site in the primers (underlined). This fragment contains the SV40 origin of bidirectional replication. The SV40-*ori* was cloned as a blunted *XbaI* fragment into several sites of *pBluescript KSII+*. Large-scale plasmid preparations were prepared from dam+ *E. coli* cells and used in the replication assay (31,61).

Cell lines and cell extract preparation for *in vitro* replication assays

The *LIGI*-deficient 46BR.1G1 cell line is a simian virus 40-transformed human cell line derived from the primary fibroblast strain 46BR expressing the defective *LIGI* enzyme (hemizygous or homozygous for the Arg-771 → Trp mutation). The complemented 46BR.1G1 pβAHLneo#2 derivative cell line also expresses a wild-type *LIGI* from a stably transfected pβAHLneo plasmid carrying a wild-type human DNA *LIGI* cDNA (17,21). Cells were cultured and extracts were prepared as previously described (63).

In vitro replication assay and replication efficiency

Reactions were performed as described (63). DNA templates (150 ng) were replicated in reactions containing final concentration of: dATP, dGTP, dTTP and dCTP, 100 μM each; GTP, UTP and CTP, 200 μM each; ATP, 4 mM; creatine phosphate (Roche), 40 mM; creatine kinase, 100 μg/ml (Roche), 1 μg SV40 T-antigen (Chimerx, Wisconsin) and 30 μl cell extracts (4 μg to 6 μg/μl). For direct analysis of the replication products, 20 μM ³²P-α-dCTP was included in the reaction mixture. Reactions were incubated for 4 h at 37°C and terminated with 50 μl of stop solution (2 μg/μl proteinase K, 2% sodium dodecyl sulfate (SDS) and 50 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0) with further incubation of 1 h at 37°C. Carrier tRNA (15 μg) was added. Replication products were ethanol-precipitated, resuspended in water and divided into two aliquots. Equal quantities of reaction products were digested with 10 units *BamHI* only or with 10 units *BamHI-DpnI* to discriminate between total and completely replicated material, respectively. Finally, digestion products were resolved by electrophoresis on 15 cm 1% agarose gels. Gels were run for 16 h at 4 V/cm in Tris-buffered EDTA buffer 1×, dried and exposed to Kodak film. Replication amounts were determined by autoradiography and quantified using ImageQuant GE Healthcare Version 1.2 software.

In vitro mutation analysis/the STRIP assay

Mutation analysis was performed using the Stability of Trinucleotide Repeats by Individual Product analysis (STRIP assay) as described before (31,63). Briefly, replication products were digested with 10 units of *DpnI* (New England Biolabs) to eliminate un-replicated parental material. *DpnI*-digested material was transformed into competent *E. coli*. Individual bacterial colonies, each representing an individual product of primate DNA replication, were picked and cultured for 6 h. Miniprep DNA was analysed for changes in repeat length by analysis of the repeat containing fragment on high-resolution 4% polyacrylamide gels. To focus upon mutation events derived from *in vitro* replication by human proteins, the background length heterogeneity within the parental template preparation and that derived through bacterial miniprep culture were determined. Background length heterogeneity for each template was determined by direct bacterial transformation of the same template DNA used for *in vitro* replication reactions, repeat length analysis and length categorization of single colonies (Fig. 6). Since the degree and distribution of length heterogeneity may vary between different bacterial preparations of a particular template, it is critical that the same template preparation used for the *in vitro* replication reactions be used for the bacterial background correction. Statistical analysis was performed between cell extracts using the χ^2 test. The observed differences were considered statistically significant only if the *P*-values were <0.05.

Transgenic mice

DM300–328 mice (C57Bl/6) transgenic for the DM1 locus were crossed with 46BR mice (129/Ola × BALB/c) carrying the 46BR.1G1 *LigI* mutation (CGG to TGG, Arg-771 to Trp)

to obtain *LigI*^{+/+} (+/+), *LigI*^{+/*m*} (+/46BR) and *LigI*^{*m/m*} (46BR/46BR) mice carrying more than 300 CTG repeats. The DM1 transgenic status was determined by PCR with the DMHR8 and DMHR9 primers (41). The 46BR mice have been described elsewhere, and the genotypes were assayed by multiplex PCR as reported (20). Housing and handling of mice were performed according to the French government ethical guidelines.

Intergenerational and somatic CTG instability in mice

Intergenerational instability was analysed by comparison of the CTG repeat lengths between transmitting parent and its offspring. DNA was extracted from the tail, collected at weaning and amplified using primers 101 and 102, and the repeat length was measured by running CTG amplification products on a 4% acrylamide denaturing gel as described previously (41). Somatic instability was determined in tissues collected from 19-month-old mice using the protocol described in Tomé *et al.* (64). Small pool-PCR (SP-PCR) amplifications and PCR product electrophoretic analyses were performed using the methods previously described (24). Briefly, DNA samples were digested with *Hind*III, and SP-PCR was performed with DM-C and DM-BR primers. SP-PCR products from mice of different *LigI* genotypes and tail/blood or tail/liver PCR products were loaded on the same 0.7% (m/v) agarose gel to compare instability between *LigI*^{+/+}, *LigI*^{+/*m*} and *LigI*^{*m/m*} mice.

CTG instability in oocytes

Mature female mice were superovulated by intramuscular injection of 5 UI of pregnant mare serum gonadotropin (PMSG: Syncro-part PMSG 500 UI from CEVA Santé) into the inguinal region followed by administration of 5 UI of human chorionic gonadotropin (HCG, Chorulon 1500 UI from Intervet) to obtain primary oocytes and follicle cells. Primary oocytes were collected from the ampullar region of the oviducts 16 h after HCG injection and freed from cumulus cells by gently pipetting briefly in 10 mg/ml hyaluronidase (Sigma H4272) at 37°C, and then washed twice in M2 medium at 37°C (Sigma M7167). DNA was extracted from oocytes of each superovulated mouse by the phenol-chloroform protocol. The size of the CTG repeat was determined by the SP-PCR protocol as described previously (24).

Statistical analyses

Statistical analyses for expansion and contraction distributions were performed with the StatView software, using the Mann–Whitney test (SAS Institute Inc.). Frequencies were analysed by Fisher's exact test. Each analysis was performed for all transmissions and was checked on groups of mice of the same age and with the same repeat sizes, to prevent bias. The observed differences were considered statistically significant only if the *P*-values were <0.05 in total transmissions and homogeneous groups.

Quantitation of *LIGI* and its phosphorylation

Proteins from young mice were extracted by mechanical homogenization in lysis buffer (0.125 M Tris–HCl pH 6.8, 4% SDS, 10% glycerol) containing complete Mini 7x protease inhibitor cocktail (Roche Applied Science) and 1 mM phenylmethyl sulfonyl fluoride (Sigma). Protein analysis of *LIGI* in mouse tissues was performed by western blot, combined with a dephosphorylation treatment with alkaline phosphatase (CIP, New England Biolabs) for 4 h at 37°C in order to assess this posttranslational protein modification. With or without dephosphorylation, samples were compared by running of the samples on 7.5% acrylamide SDS-polyacrylamide gel electrophoresis gel, transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with 5H5 monoclonal antibody against *LIGI* (5H5, MBL; 1:5000, ± 125 kDa). Loading was normalized for the same amount of protein in each lane. Actin, 42 kDa (Ab-5, BD Biosciences; 1:5000) was used as a control. The functional protein extracts from 46BR.1G1 cell extracts were prepared as described in López Castel *et al.* (15).

Conflict of Interest statement. None declared.

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