

Mutator Phenotype of *Caenorhabditis elegans* DNA Damage Checkpoint Mutants

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ABSTRACT

DNA damage response proteins identify sites of DNA damage and signal to downstream effectors that orchestrate either apoptosis or arrest of the cell cycle and DNA repair. The *C. elegans* DNA damage response mutants *mrt-2*, *hus-1*, and *clk-2(mn159)* displayed 8- to 15-fold increases in the frequency of spontaneous mutation in their germlines. Many of these mutations were small- to medium-sized deletions, some of which had unusual sequences at their breakpoints such as purine-rich tracts or direct or inverted repeats. Although DNA-damage-induced apoptosis is abrogated in the *mrt-2*, *hus-1*, and *clk-2* mutant backgrounds, lack of the apoptotic branch of the DNA damage response pathway in *cep-1/p53*, *ced-3*, and *ced-4* mutants did not result in a Mutator phenotype. Thus, DNA damage checkpoint proteins suppress the frequency of mutation by ensuring that spontaneous DNA damage is accurately repaired in *C. elegans* germ cells. Although DNA damage response defects that predispose humans to cancer are known to result in large-scale chromosome aberrations, our results suggest that small- to medium-sized deletions may also play roles in the development of cancer.

THE genome is under continuous surveillance for DNA damage by checkpoint sensor proteins, which, if activated, can elicit temporary arrest of the cell cycle and repair of the damaged lesion. In multicellular eukaryotes, an alternative fate for cells that reach a critical threshold of DNA damage is apoptosis. Malfunction of DNA damage response proteins can stimulate tumorigenesis in humans (VENKITARAMAN 2002), probably as a consequence of improper processing of endogenous or exogenous forms of DNA damage that results in alterations to the genome. An understanding of the precise relationship between genome instability and the development of cancer is currently a topic of intense study and includes analysis of gross chromosomal rearrangements (GCRs) for many forms of cancer. While GCRs are commonly observed in cancer cells and may contribute to tumorigenesis, it is unclear if they herald the presence of other forms of DNA damage that are also relevant. We have chosen to address this issue by studying DNA damage response mutants in the nematode *Caenorhabditis elegans*.

Proteins that respond to DNA damage are thought to function via a tiered network of biochemical interactions. Putative sensors of DNA damage such as the

phosphatidylinositol-3 (PI-3)-like kinases ATR and ATM and their respective partners ATRIP and the MRE11/RAD50/NBS1 complex help to initiate a DNA damage response (DDR) (SANCAR *et al.* 2004). A complex of checkpoint proteins that is independently recruited to sites of DNA damage is the RAD9/RAD1/HUS1 (9-1-1) proliferating cell nuclear antigen (PCNA)-like sliding clamp, which is loaded onto single-stranded DNA by the RAD17 clamp loader and its four replication factor C subunits (GRIFFITHS *et al.* 1995; KOSTRUB *et al.* 1998; BURTELOW *et al.* 2000; CASPARI *et al.* 2000; ZOU *et al.* 2002; BERMUDEZ *et al.* 2003). The ATR and ATM checkpoint kinases respond to DNA damage by phosphorylating members of the 9-1-1 damage sensor complex as well as downstream “mediators” such as BRCA1, CLASPIN, 53BP1, the signaling kinases CHK1 and CHK2, and effectors such as p53 (SANCAR *et al.* 2004).

Genetic studies in mice and yeast suggest that DNA damage response proteins may suppress genome instability, in part, by facilitating the repair of endogenous DNA double-strand breaks (DSBs) (PATEL *et al.* 1998; MOYNAHAN *et al.* 1999, 2001; DENG and SCOTT 2000; MYUNG *et al.* 2001a,b; HOWLETT *et al.* 2002; KRAAKMAN-VAN DER ZWET *et al.* 2002; VENKITARAMAN 2002; D’ANDREA and GROMPE 2003; PENNANEACH and KOLODNER 2004). Examination of genome stability in haploid yeast mutants defective for the 9-1-1 checkpoint complex has revealed modestly elevated levels of GCRs

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(MYUNG *et al.* 2001b). Mutation of vertebrate 9-1-1 complex subunits results in genome instability and lethality (WEISS *et al.* 2000; BUDZOWSKA *et al.* 2004; HOPKINS *et al.* 2004; KOBAYASHI *et al.* 2004). Deficiency for mediators of the DNA damage response such as BRCA1 or BRCA2 also results in lethality accompanied by translocations, loss of chromosome arms, and aneuploidy (VENKITARAMAN 2002). In contrast, mutation of p53 in mice does not lead to genome instability or a Mutator phenotype (NISHINO *et al.* 1995; BUETTNER *et al.* 1997; RELIENE and SCHIESTL 2003). Rather, p53 is thought to suppress cancer by acting to control cell proliferation via either apoptosis or senescence (LOWE *et al.* 2004).

C. elegans displays tissue-specific responses to DNA damage. Checkpoint proteins such as the 9-1-1 complex or ATM/ATR can initiate apoptosis in meiotic germ cells at the pachytene stage or cell cycle arrest in the mitotic germ cells (GARTNER *et al.* 2004; GARCIA-MUSE and BOULTON 2005), whereas somatic cells are refractory to these cellular responses to DNA damage (GARTNER *et al.* 2000). Mammalian p53 responds to DNA damage by eliciting either apoptosis or cell cycle arrest (ATTARDI 2005). However, *C. elegans* and *Drosophila* orthologs of p53 affect only the induction of DNA-damage-induced apoptosis (DERRY *et al.* 2001; SCHUMACHER *et al.* 2001; BRODSKY *et al.* 2004), suggesting that the mammalian cell cycle arrest function may be derived.

Deficiency for the *C. elegans* 9-1-1 complex subunits HUS-1 or MRT-2 results in defective responses to both ionizing radiation (IR)-induced apoptosis and cell cycle arrest in germ cells (AHMED and HODGKIN 2000; GARTNER *et al.* 2000; HOFMANN *et al.* 2002). Mutations in a third *C. elegans* DNA damage checkpoint gene, *clk-2*, also abrogate the apoptotic and cell cycle arrest responses to IR and confer an additional defect in the S-phase DNA replication checkpoint (AHMED *et al.* 2001). *clk-2* has recently been shown to function downstream of *atl-1*, a homolog of the PI-3-like DNA-damage-signaling kinase ATR that interacts with DSBs (GARCIA-MUSE and BOULTON 2005). Both *atl-1* and *clk-2* are essential, and a strong defect in either gene results in the accumulation of single-stranded DNA and mitotic failure (GARCIA-MUSE and BOULTON 2005). Two conditional mutations of *clk-2* have been identified: *mn159* was recovered in a screen for radiation-hypersensitive mutants of *C. elegans* (hence its former gene name “*rad-5*”) (HARTMAN and HERMAN 1982), and *qm37* was identified in a screen for mutations that confer a maternally rescued Slow Growth phenotype (LAKOWSKI and HEKIMI 1996).

Here we show that mutations in the *C. elegans* DDR genes *mrt-2*, *hus-1*, and *clk-2* result in an elevation in the frequency of spontaneous mutation, whereas defects in genes required exclusively for DNA-damage-induced apoptosis do not. Mutations that result from DDR defects are often small- to medium-sized deletions, suggesting a failure to repair spontaneous lesions that result in strand breaks.

MATERIALS AND METHODS

Strains: All strains were cultured at 20° as described previously (SULSTON and HODGKIN 1988). Strains used in this study were Bristol N2 wild type, *hus-1(op241)* I, *dpy-5(e61)* I, *cep-1(lg12501)* I, *cep-1(gk138)* I, *dpy-17(e164)* III, *ced-4(n1162)* III, *clk-2(mn159)* III, *clk-2(qm37)* III, *unc-32(e191)* III, *mrt-2(e2663)* III, *unc-17(e2754)* IV, *ced-3(n717)* IV, *unc-60(e2763)* V, *him-7(e1480)* V, *unc-46(e177)* V, *vab-8(e2764)* V, *lon-2(e2775)* X, *unc-58(e665)* X, 18 *unc-58(e665)* suppressor mutants numbered *unc-58(e2815)* X to *unc-58(e2832)* X, and *ypIs1* [*rol-6(su1006)* *mrt-2(+)*].

Genetics: All marker strains were outcrossed five times *vs.* Bristol N2 wild type before use. The presence of *mrt-2* or *hus-1* was assessed by scoring for sterility following gamma irradiation of six L1 larvae per strain at a dose of 60 Gy. The presence of *clk-2(mn159)* was assessed on the basis of its temperature-sensitive sterile phenotype (HARTMAN and HERMAN 1982). The mutation *hus-1(op241)*, which lies on chromosome I, was originally identified in the strain CB1480 *him-7(e1480)* V (GARTNER *et al.* 2000). The *hus-1(op241)* and *him-7(e1480)* mutations were separated by crossing them into and out of *unc-46* V and *dpy-5* I backgrounds, respectively. Two F₂ lines each of the *hus-1(op241);unc-58(e665)* and *him-7(e1480);unc-58(e665)* lines were established. Two *mrt-2* F₂ lines were established after six and seven outcrosses with N2 wild type, both were crossed with *unc-58(e665)*, and two *mrt-2;unc-58(e665)* F₂ lines each were established for the *mrt-2* parental lines. *clk-2(mn159)* was outcrossed four times with N2 wild type, crossed into and out of backgrounds containing the flanking genetic markers *dpy-17* and *unc-32*, and crossed an additional three times *vs.* *dpy-17;unc-32*; two *clk-2(mn159)* strains, each used to generate independent *clk-2(mn159);unc-58(e665)* strains, were established. *unc-30;unc-58(e665)* and *dpy-17;unc-58(e665)* were used to generate *ced-3;unc-58(e665)* and *ced-4;unc-58(e665)* doubles, respectively. For rescue of *mrt-2*, an extrachromosomal array containing a wild-type copy of the *mrt-2* gene and a *rol-6(su1006)* Dm marker was integrated into a single locus in the genome by irradiating L4 larvae with 20 Gy IR to create the insertion *ypIs1*. This rescuing transgene was crossed with three independent lines of *mrt-2;unc-58(e665)* to generate *mrt-2;unc-58(e665);ypIs1* strains.

Mutator assays: To confirm the presence of spontaneous visible mutants that appeared in the *mrt-2* mutant background, L4 larvae with unusual phenotypes were singled and their progeny were scored for transmission of the parental phenotype. For sterile mutants, nonsterile siblings were picked and their progeny were scored for segregation of the sterile phenotype.

For *unc-58(e665)* assays, OP50 bacteria was spread over the entire surface of 50 or 60 mm NGM plates and allowed to dry for 2 days. For each assay, a total of 40 plates were seeded with five *unc-58(e665)* L4 larvae each per genotype. One to eight additional trials were conducted using independently derived strains to assess mutation frequency (HODGKIN 1974). Plates were allowed to grow for a month until starvation, and the agar from each plate was then transferred to a 90-mm NGM plate with a streak of bacteria to one side. The presence of suppressor mutations was assessed 3 and 4 days later, and a single L4 larva was picked for each suppressor.

The five *unc-58(e665)* larvae used to seed each plate gave rise to ~150 F₁ progeny apiece, corresponding to 1500 haploid genomes. However, *unc-58(e665)* moves very slowly and brood size subsequently drops as a consequence of starvation and overcrowding, so 750 F₁ might give rise to 30 F₂ progeny each, on average, for a total of 22,500 F₂ animals. The mean number of animals on a starved *unc-58(e665)* plate was 57,000 ± 7000 for 50-mm plates (Table 2) or 82,200 ± 10,500 for 60-mm

plates (Table 3) ($n = 16$). For 50-mm plates, an additional 34,500 progeny would arise from F_2 , F_3 , and F_4 parents, each of which were estimated to produce 15 progeny apiece, on average, accounting for 2300 parents or an additional 4600 haploid genomes. Thus, the total number of haploid genomes sampled per 50-mm plate was estimated as 6100. For parents that produce 15 progeny apiece, there is a 1.8% probability that none of the progeny of a heterozygous parent will be homozygous for the mutation, which lowers the estimated number of haploid genomes sampled per 50-mm plate to 6020. Similar calculations for the 60-mm plates used in Table 2 predict 9320 haploid genomes per plate.

Deletion analysis: Genomic DNA was prepared from suppressors using a Puregene cell and tissue kit (Gentra, Minneapolis), as recommended. Primers spanning each mutant locus were spaced ~1 kb apart and amplified using the polymerase chain reaction (PCR). Primers were designed such that the ends of adjacent PCR products overlapped, except for the *lin-15* locus, where nonoverlapping primers spaced 10 kb apart were also used. Deletions were identified on the basis of the change in size of a PCR product or on the absence of one or more PCR products and the ability to generate a novel PCR product from mutant, but not wild-type, template DNA using primers flanking predicted breakpoints.

G-tract PCR: For template DNA, 10 *C. elegans* adults were mixed with 10 μ l of lysis buffer (50 mM KCl, 10 mM, Tris pH 8.3, 2.5 mM MgCl₂, 0.45% NP-40, 0.45% Tween-20, 0.01% gelatin) and 2 μ l of 10 mg/ml proteinase K, incubated at 60° for 60 min and then at 95° for 25 min. One microliter of template DNA was then added to 24 μ l of PCR reaction containing 50 mM LiCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 0.1 mM MgCl₂, 2 mM dNTPs, 5–10 μ M G-tract primers, and 5 units/ μ l Taq DNA polymerase and incubated at 94° for 2 min, followed by 34 cycles of 94° for 45 sec, 60° for 30 sec, 72° for 90 sec and finally a 72° 5-min extension. Li⁺ was used instead of K⁺ as the monovalent cation in the 10 \times PCR buffer, as stable G-quartets that can inhibit DNA polymerases may form at long G-tracts in the presence of K⁺ (WOODFORD *et al.* 1994). Primer sets were F55F3 (GCCAATCTATCAAACTCT GACTG and CGACCAAGGTTCTATCATACGAA), F38A6 (GA ACCACTTCTGGGACCTCCG and TGGCTGGGCTGATGTAG TTCC), and Y41E3 (ACCGTTTCGAAACATGTTGCCAA and TGTATGCCATTTACTAACTCTCC). Template DNA was prepared from strains grown at 20° and 25° to determine if growth temperature might affect the frequency of deletion, but identical results were obtained for all primer sets.

Life-span analysis: A total of 100 L4 larvae of each genotype were used to initiate life-span assays. Adults were aged in groups of six, as described (MEIER *et al.* 2006).

RESULTS

Elevated levels of spontaneous mutation in *C. elegans*

DDR mutants: Several spontaneous visible mutations were noted when propagating the *C. elegans* DNA damage response mutant *mrt-2* (AHMED and HODGKIN 2000). To confirm this observation, 40 freshly outcrossed F_3 *mrt-2* founders were singled, most of which failed to segregate visible mutations in the F_4 generation. However, when progeny of 680 F_4 *mrt-2* hermaphrodites that had F_3 siblings with no visible phenotypes were examined, 11 visible mutants were identified: 4 Uncoordinated (Unc), 2 Dumpy (Dpy), 2 Multivulva, 1 Long, and 1 Variable (Vab), whereas no spontaneous

visible mutants were observed for progeny of 940 control *mrt-1* hermaphrodites.

Spontaneous mutations occur rarely in the wild-type *C. elegans* strain Bristol N2 (ANDERSON 1995). To determine if *mrt-2* might confer a Mutator phenotype, a *mrt-2* strain carrying a rescuing extrachromosomal array containing the wild-type *mrt-2* gene was compared with siblings from the same strain that had lost the rescuing array and were therefore checkpoint defective (AHMED and HODGKIN 2000). The progeny of 780 *mrt-2* mutants carrying the rescuing array failed to segregate visible mutations, whereas a total of 10 visible mutations were identified among the progeny of 780 nonrescued sibling *mrt-2* individuals: 2 Unc, 1 Vab, 3 sterile Dpy, 2 egg-laying defective, 1 protruding Vulva, and 1 gonad development abnormal. Thus, the *mrt-2* DDR gene suppresses the frequency of spontaneous mutation.

Inspection of the spontaneous *mrt-2*-induced visible mutants suggested candidate genes, and linkage and complementation tests demonstrated that alleles of *dpy-31* (NOVELLI *et al.* 2004), *lon-2*, *unc-17*, *unc-60*, *vab-8*, and *lin-15* had been isolated (BRENNER 1974; HORVITZ *et al.* 1983). A *lin-15* Multivulva phenotype arises only when the functions of two neighboring genes, *lin-15a* and *lin-15b*, have been eliminated (HUANG *et al.* 1994). Thus, isolation of a *lin-15* mutation conferring a Multivulva phenotype from a *mrt-2* background suggested that a genome rearrangement may have occurred. PCR analysis of the *lin-15* locus amplified the expected products from N2 wild-type genomic DNA but not from that of the *lin-15* mutant, whereas control loci on a different chromosome were intact for both strains. Further experimentation revealed that a deletion extending 165 kb to the right and 67 kb to the left of the *lin-15* locus had occurred. Nested PCR reactions using primers flanking the putative breakpoints failed to yield a PCR product, suggesting a complex genome rearrangement. Analysis of four other *mrt-2*-induced mutations revealed that small- to medium-sized deletions had occurred: 0.15 kb for *unc-60(e2763)* (McKIM *et al.* 1994), 0.435 kb for *lon-2(e2775)* (<http://www.wormbase.org>), 1.813 kb for *unc-17(e2754)* (RAND 1989), and 3.436 kb for *vab-8(e2764)* (WOLF *et al.* 1998). For two of these four deletions, a tract of G-rich sequence was adjacent to one breakpoint (Table 1, Figure 1). Such sequences occur rarely in the *C. elegans* genome (Figures 1 and 4), suggesting the involvement of the 9-1-1 DDR complex in resolving G-rich secondary structures (CHEUNG *et al.* 2002). The sixth visible mutant isolated from *mrt-2* was the founding *dpy-31* allele, *e2770*, which was sequenced and shown to result from a T-to-C transition (NOVELLI *et al.* 2004).

The *clk-2* checkpoint gene functions in the *mrt-2* DDR pathway and also in a parallel DDR pathway (AHMED *et al.* 2001). A spontaneous mutation in *dpy-10* was isolated from the *clk-2(mn159)* mutant background, and analysis of the *dpy-10* locus (LEVY *et al.* 1993)

TABLE 1
Breakpoints of spontaneous *mrt-2* and *clk-2(mn159)* deletion products

<p><i>lon-2(e2755) (mrt-2)</i></p> <p>AAACATAAATCTTTTTGTCATGCCATTTTTGGAAA..7073..gtcgtaaatttcattacacacggcgttctctttt <u>AT</u> (insert) caggtgagttaaaaatatttgggagggggggggggg..6623..<u>AT</u>CATAGAAAACGTTTTTGTAAATTTTTTAAAT (polypurine tract)</p>
<p><i>unc-17(e2754) (mrt-2)</i></p> <p>TTTAAAAATTTAAAAAAGGCTCTGAACCTATTTTTT..24834..<u>ggatt</u>tttttttgattttttacaacggaaaaatt cgcggggagtgggggggggggggggggggggggg..26647..<u>GGATT</u>TGCTACCGGTTGTGCAAAAAGGAGTTCAA (polypurine tract)</p>
<p><i>unc-60(e2763) (mrt-2)</i></p> <p>AAAAATTTCCATGTATTTCCACATTCCACTTCCCA..11548..<u>aactcct</u>atatttcagctgccagacaacgccccag AAgtacaggcctccgagatgttcggacctcgacgag..11690..<u>AAG</u>AGCGTGAAGAGCGACCTGATGTCCAACCAAAG (hairpin)</p>
<p><i>vab-8(e2764) (mrt-2)</i></p> <p>CACACCGTTTTCTTCTCGAGACCACCCATAAAAGT..26399..<u>cg</u>gggtggcgcaaccagaaataactgtcgcccgct (hairpin) tcaatcaaatactgatatggatgtttctgaaatgg..29834..<u>GT</u>TACCGGAGAGCAAGCCATTTGGAAGAATATGC (hairpin)</p>
<p><i>dpy-10(e2760) (clk-2 mn159)</i></p> <p>TTAATGAAAAATTTAAACCCTAGAGCTTGAAAAT..4183..<u>cca</u>caaacgcagcttcttgagaaaaattaattgg CTAAACAAATAAAACAATAAAAAAACTAAAAATAAAAA (insert) acaatgtagatgagaagagaagtactacggttaggt..7799..<u>TGAGTGAG</u>TGTGTGGATCGAAAAGTGAAATTTCAA<u>GTGAGC</u> GGGGGCATAGGGTGGTGGCGGCGCCGCGCGCGC (direct repeat and G/C-rich tract)</p>

Sequences present at the breakpoints are in uppercase. Deleted sequences are in lowercase. Positions of breakpoints relative to the cosmid sequence for each gene are indicated (<http://www.wormbase.org>). Microhomology at breakpoints is underlined and in italics. Novel DNA sequence inserted between each breakpoint during NHEJ is indicated. DNA sequences with unusual qualities are underlined, in boldface, and defined in parentheses beneath the sequence. A direct repeat at the *e2760* breakpoint is italicized, underlined, and in boldface.

revealed a 3.616-kb deletion and a long 42-bp G/C-rich sequence located near one deletion breakpoint (Table 1, Figure 1). Thus, five of six spontaneous *mrt-2*-induced mutations and one spontaneous *clk-2*-induced mutation resulted from deletions, three of which displayed G- or G/C-rich sequences near one deletion breakpoint.

IR hypersensitivity of checkpoint mutants: Given that both *mrt-2* and *clk-2(mn159)* appeared to be Mutators on the basis of the isolation of visible mutations, multiple outcrosses were performed to remove extraneous mutations from strains used in this study. Prior to quantifying their effects on the frequency of spontaneous mutation, the severity of the checkpoint defects of the DDR mutations was assessed. Although *mrt-2* and *hus-1* physically interact and encode members of the heterotrimeric 9-1-1 PCNA-like sliding clamp (BOULTON *et al.* 2002), the *mrt-2(e2663)* mutation is a null allele that is fully defective for the G2/M DNA damage checkpoint and for telomere replication, whereas *hus-1(op241)* is a partial loss-of-function mutation that has weaker checkpoint defects and is proficient for telomere replication (AHMED and HODGKIN 2000; HOFMANN *et al.* 2002). The

clk-2(mn159) and *clk-2(qm37)* mutations confer radiation response defects that are somewhat weaker than those of *hus-1(op241)*, but both *clk-2* alleles have additional S-phase checkpoint defects and fail to respond to DNA replication forks that stall as a consequence of hydroxyurea-mediated nucleotide depletion (AHMED *et al.* 2001). The magnitude of the DNA damage response defects of outcrossed DDR mutant strains was *mrt-2 > hus-1 > clk-2(mn159)*, as assessed by measuring relative survival of progeny when L4 larvae were treated with gamma irradiation (Figure 2A). Previous results indicated that the germlines of *clk-2(qm37)* display a mildly enhanced radiation hypersensitivity phenotype in comparison with those of *clk-2(mn159)* (AHMED *et al.* 2001). These results were recapitulated with isogenic versions of the *clk-2* alleles, and both *clk-2(qm37)* homozygotes and *clk-2(mn159)/clk-2(qm37)* trans-heterozygotes were more sensitive to IR than *clk-2(mn159)* homozygotes (Figure 2, B and C). The radiation hypersensitivity of the germlines of the above mutants provided a measure of the relative magnitude of their DDR defects, and it seemed plausible that their frequencies of spontaneous mutation might scale accordingly.

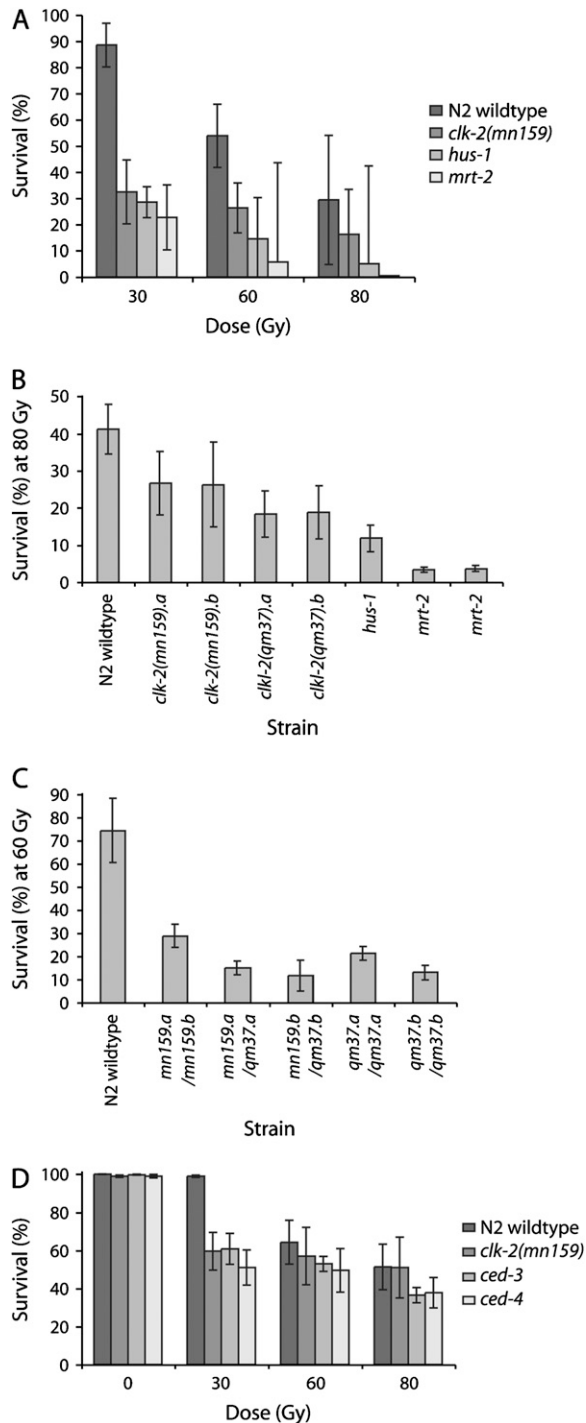


FIGURE 2.—Radiation hypersensitivity of checkpoint mutants. (A) Dose-response analysis of radiation hypersensitivity. Comparisons of IR hypersensitivity of *clk-2* mutant strains that were independently outcrossed (*a* and *b*) at doses of (B) 80 Gy and (C) 60 Gy. (D) Apoptosis removes germ cells with lethal levels of DNA damage. For all panels, progeny derived from irradiated L4 larvae were scored for embryonic lethality ($n = 5$ broods scored/strain). Percentage of survival was normalized relative to unirradiated controls and standard deviations are shown.

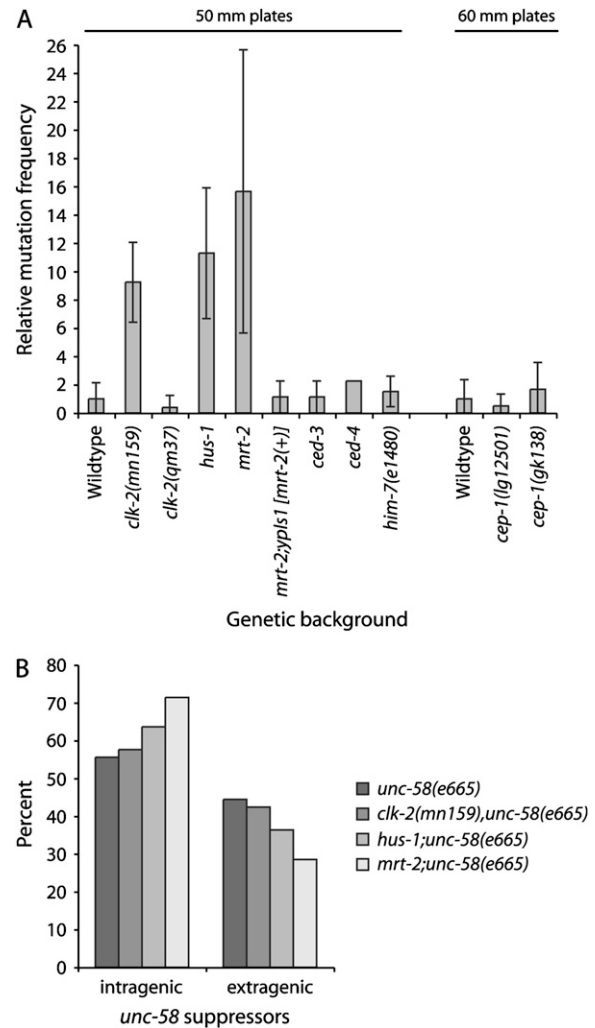


FIGURE 3.—Frequencies of spontaneous mutation as determined using *unc-58(e665)*. (A) Relative levels of spontaneous mutation in various *unc-58(e665)* genetic backgrounds. For each experiment, 40 50-mm plates were scored for the strains *unc-58(e665)*, *clk-2(mn159);unc-58(e665)*, *clk-2(qm37);unc-58(e665)*, *hus-1(op241);unc-58(e665)*, *mrt-2;unc-58(e665)*, *mrt-2;unc-58(e665);ypIs1[rol-6(su1006) mrt-2(+)]*, *ced-3;unc-58(e665)*, *ced-4;unc-58(e665)*, and *him-7(e1480);unc-58(e665)*. Additional results obtained using 60-mm petri dishes are shown for *cep-1/p53* mutants. *unc-58(e665)* assays were repeated for control, *mrt-2*, *hus-1(op241)*, and *clk-2(mn159)* genetic backgrounds with similar results. (B) Relative percentage of total intragenic vs. extragenic *unc-58* suppressors. Note that wild-type suppressors were rare, which violates some assumptions of the z-test for proportions.

of *clk-2* mutant germlines was similar to that of *ced-3* and *ced-4* (Figure 2D), the strong radiation-induced apoptosis defects of the *clk-2* alleles may account for their hypersensitivity to IR (AHMED *et al.* 2001). While apoptosis did clear the germline of some nuclei with critical levels of DNA damage (Figure 2D) (BHALLA and DERNBURG 2005), deficiency for *cep-1/p53*, *ced-3*, or *ced-4* had no effect on the frequency of spontaneous *unc-58(e665)* revertants (Figure 3A, Tables 2 and 3). These results agree with the lack of a Mutator phenotype for

TABLE 2
unc-58(e665) spontaneous revertant frequencies

<i>unc-58(e665)</i> background	Trial	Plates with revertants/ total plates	$f(0)$	Mutation frequency (revertants/gamete)	Spontaneous mutations/gamete
Wild type	1	2/40	0.95	8.56×10^{-6}	
	2	0/40	1	0	
	3	0/40	1	0	
	4	0/40	1	0	
	5	1/40	0.975	8.88×10^{-6}	
	6	1/40	0.975	8.88×10^{-6}	
	7	0/40	1	0	
	8	0/40	1	0	
	9	1/40	0.975	8.88×10^{-6}	$3.9 \pm 4.4 \times 10^{-6}$
<i>clk-2(mn159)</i>	1	6/40	0.85	5.7×10^{-5}	
	2	5/40	0.875	4.69×10^{-5}	
	3	5/40	0.875	4.69×10^{-5}	
	4	3/40	0.925	2.74×10^{-5}	
	5	3/40	0.925	2.74×10^{-5}	
	6	3/40	0.925	2.74×10^{-5}	
	7	3/40	0.925	2.74×10^{-5}	
	8	4/40	0.9	3.7×10^{-5}	
	9	3/40	0.925	2.74×10^{-5}	$3.6 \pm 1.1 \times 10^{-5}$
<i>clk-2(qm37)</i>	1	0/40	1	0	
	2	0/40	1	0	
	3	0/40	1	0	
	4	0/40	1	0	
	5	0/40	1	0	
	6	1/40	0.975	8.88×10^{-6}	$1.5 \pm 3.3 \times 10^{-6}$
<i>hus-1</i>	1	7/40	0.825	6.75×10^{-5}	
	2	6/40	0.85	5.7×10^{-5}	
	3	4/40	0.9	3.7×10^{-5}	
	4	2/40	0.95	1.8×10^{-5}	
	5	3/40	0.925	2.74×10^{-5}	
	6	6/40	0.85	5.7×10^{-5}	$4.4 \pm 1.8 \times 10^{-5}$
<i>mrt-2</i>	1	12/40	0.7	1.25×10^{-4}	
	2	6/40	0.85	5.7×10^{-5}	
	3	8/40	0.8	3.7×10^{-5}	
	4	5/40	0.875	1.8×10^{-5}	
	5	9/40	0.775	2.74×10^{-5}	
	6	10/40	0.75	1.0×10^{-4}	$6.1 \pm 3.9 \times 10^{-5}$
<i>mrt-2;ypIs1</i>	1	0/40	1	0	
	2	1/40	0.975	8.88×10^{-6}	$4.4 \pm 4.4 \times 10^{-6}$
<i>ced-3</i>	1	1/40	0.975	8.88×10^{-6}	
	2	0/40	1	0	$4.4 \pm 4.4 \times 10^{-6}$
<i>ced-4</i>	1	1/40	0.975	8.88×10^{-6}	
	2	1/40	0.975	8.88×10^{-6}	$8.8 \pm 0.0 \times 10^{-6}$
<i>him-7</i>	1	1/40	0.975	8.88×10^{-6}	
	2	1/40	0.975	8.88×10^{-6}	
	3	0/40	1	0	$5.9 \pm 4.2 \times 10^{-6}$

The frequency of spontaneous germline mutations conferring suppression of *unc-58(e665)* was assessed using the Poisson equation to account for the possibility that more than one reversion event may have occurred on a single plate on the basis of the number of plates that lacked reversion events. The general Poisson equation for n (not necessarily) = 0 is $p(n) = (m^n e^{-m}) / n!$, which reduces to $f(0) = e^{-m}$ for $n = 0$, where $f(0)$ is the fraction of plates from a given trial that did not contain an *unc-58(e665)* revertant. Mutation frequencies were calculated as: (m , the mean number of spontaneous mutations per plate) / 6020 (= number of haploid genomes per plate) (ZALEVSKY *et al.* 1999). See MATERIALS AND METHODS for an estimation of the number of haploid genomes per 50-mm plate used in Table 2. Standard deviations are shown for mean mutation frequencies.

TABLE 3
unc-58(e665) spontaneous revertant frequencies for *cep-1* mutants

<i>unc-58(e665)</i> background	Trial	Plates with revertants/ total plates	$f(0)$	Mutation frequency (revertants/gamete)	Spontaneous mutations/gamete
Wild type	1	2/40	0.95	5.52×10^{-6}	
	2	0/40	1	0	
	3	0/40	1	0	
	4	0/40	1	0	
	5	1/40	0.975	2.73×10^{-6}	
	6	0/40	1	2.73×10^{-6}	
	7	1/40	0.975	0	
	8	0/40	1	0	$1.4 \pm 1.9 \times 10^{-6}$
<i>cep-1(lg12501)</i>	1	1/40	0.975	2.73×10^{-6}	
	2	0/40	1	0	
	3	1/40	0.975	2.73×10^{-6}	
	4	0/40	1	0	
	5	0/40	1	0	
	6	0/40	1	0	
	7	0/40	1	0	
	8	0/40	1	0	$0.68 \pm 1.2 \times 10^{-6}$
<i>cep-1(gk138)</i>	1	2/40	0.95	5.52×10^{-6}	
	2	0/40	1	0	
	3	1/40	0.975	2.73×10^{-6}	
	4	2/40	0.95	5.52×10^{-6}	
	5	0/40	1	0	
	6	0/40	1	0	$2.3 \pm 2.5 \times 10^{-6}$

Calculations were made as for Table 2, except that 60-mm plates were used, yielding an estimated 9320 haploid genomes sampled per plate.

clk-2(qm37), which is deficient for DNA-damage-induced apoptosis (Figure 3A, Table 2). Similarly, studies of p53 mouse mutants have indicated that defects in DNA-damage-induced apoptosis do not affect the frequency of spontaneous mutation in mammals (NISHINO *et al.* 1995; BUETTNER *et al.* 1997; RELIENE and SCHIESTL 2003).

hus-1(op241) was originally identified in a genetic background containing *him-7(e1480)*, which confers a weak chromosome-loss phenotype suggestive of a defect in genome stability (HODGKIN *et al.* 1979; GARTNER *et al.* 2000). As a control, we determined the effect of *him-7* on the frequency of spontaneous mutation. *him-7* was separated from the *hus-1(op241)* mutation, and *him-7*; *unc-58(e665)* double mutants were constructed. *him-7* displayed a frequency of spontaneous mutation similar to that of wild type (Figure 3A, Table 2), indicating that it plays little or no role in the repair of endogenous DNA damage. In agreement with this possibility, *him-7* germlines displayed normal sensitivity to ionizing radiation (data not shown).

Molecular analysis of *unc-58* mutations: Given that most of the spontaneous visible mutations isolated from the *mrt-2* and *clk-2(mn159)* backgrounds were deletions, the molecular nature of intragenic *unc-58* mutations from the Mutator assays was determined. Eleven/twelve, 3/5, and 12/19 intragenic *unc-58* revertants derived

from *mrt-2*, *hus-1*, and *clk-2(mn159)* backgrounds, respectively, were deletions (Figure 4; data not shown). In contrast, *clk-2(qm37)* and wild-type backgrounds did not give rise to deletions ($n = 5$ and 6, respectively), as expected from numerous spontaneous *C. elegans* mutations that have been previously isolated and sequenced (PULAK and ANDERSON 1988). Thus, checkpoint defects can trigger an increase in the frequency of spontaneous deletions. cDNA sequencing has defined long and short transcripts from the *unc-58* locus, where the longer transcription product contains two additional 5' exons (Figure 4A) (<http://www.wormbase.org>). However, all of the intragenic *unc-58(e665)* deletions that were recovered eliminated segments of the short *unc-58* transcript, suggesting that it may be responsible for the dominant Unc phenotype of *unc-58(e665)*.

Deletions can result from DNA double-strand breaks that are healed either by homology-driven recombination or by nonhomologous end-joining (NHEJ). Canonical NHEJ seals breaks that have little or no homology via proteins that include DNA ligase IV, XRCC4/Lif1, and the Ku heterodimer (LIEBER *et al.* 2003). An alternative noncanonical NHEJ pathway known as microhomology-mediated end-joining relies either on a short patch of microhomology or on insertion of short DNA sequences to seal a DSB (FELDMANN *et al.* 2000; MA *et al.* 2003; YU and GABRIEL 2003; McVEY

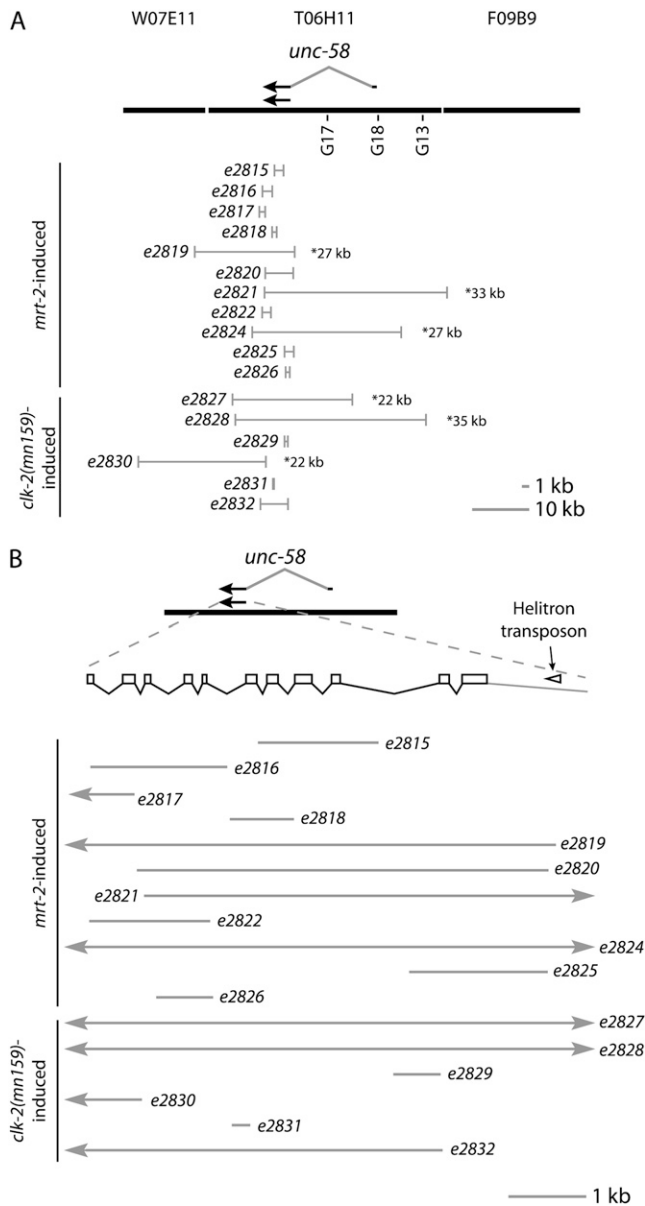


FIGURE 4.—Models of *unc-58* deletions induced in checkpoint-defective backgrounds. (A) Large-scale model of the genome region surrounding *unc-58* (<http://www.wormbase.org>). Alternative *unc-58* transcripts and G-tracts are shown above or below the cosmid sequence, respectively. Deletions are indicated as shaded bars with allele designations to the left and approximate lengths of large deletions to the right. (B) Enlargement of the smaller *unc-58* transcription unit. The nonautonomous Helitron transposon is indicated as an open arrowhead. Deletions are indicated as shaded bars if their breakpoints fell within the enlarged region or as shaded bars with arrowheads for breakpoints that occurred upstream or downstream of the area shown.

et al. 2004b). The breakpoints of 11 *mrt-2* and 6 *clk-2(mn159)* deletions of the *unc-58* locus were mapped using PCR and sequenced. Deletions from both mutant backgrounds primarily consisted of either small deletions between 0.5 and 2 kb or large deletions between 10 and 35 kb (Figure 4). Molecular analysis of the break-

points of these *unc-58* deletions revealed 7- to 47-bp insertions at breakpoints for 3/6 deletions from the *clk-2(mn159)* background and 1- to 199-bp insertions at breakpoints for 4/11 deletions from the *mrt-2* background. In addition, short patches of microhomology >1 nucleotide were apparent at breakpoints for 3/6 *clk-2(mn159)* deletions and for 4/11 *mrt-2* deletions (Tables 4 and 5). Thus, microhomology-mediated end-joining may be responsible for many of the recovered deletions, which would be consistent with observations that *lig-4*-mediated NHEJ is repressed in the *C. elegans* germline (MARTIN *et al.* 2005; CLEJAN *et al.* 2006).

Studies of deletions that result from NHEJ of DSBs induced at specific sites in the genome have revealed that one end of the DSB can be protected from degradation while the other end is resected, which may aid the search for regions of microhomology that can be used for end-joining (YU and GABRIEL 2003). Therefore, the breakpoints of *unc-58* deletions were examined for sequences that might explain spontaneous DSB induction. Several deletions that occurred in strains deficient for either *mrt-2* or *clk-2* displayed unusual qualities at their breakpoints. The *clk-2(mn159)*-induced *e2828* deletion contained three direct AATTTTCATTCCG TGA repeats at one breakpoint, where the breakpoint occurred between the first and second repeats (Table 4). Similarly, the *mrt-2*-induced *e2824* deletion had two direct GAAAAAAAAATCAAA repeats at one breakpoint, which occurred between the direct repeats, and the *mrt-2*-induced *e2816* deletion had a 30-bp polypurine-rich tract adjacent to one breakpoint (Table 5). Such sequences have been proposed to form unusual structures during DNA replication that may trigger replication fork collapse and DSB formation (BACOLLA *et al.* 2004).

Two groups of deletions had breakpoints that occurred near one another. The first group involved *e2816*, *e2818*, *e2822*, *e2826*, and *e2831*, all of which had one breakpoint that occurred within a 350-bp interval. However, no obvious sequence anomalies were recognized in this area, and the *e2816* deletion had an unusual and potentially unstable polypurine sequence located at its other breakpoint. The second group of deletions, *e2819*, *e2820*, and *e2825*, all had one breakpoint located within a 240-bp repetitive element that corresponds to a nonautonomous Helitron transposon, which is thought to transpose via rolling circle replication (Figure 4B) (KAPITONOV and JURKA 2001). One breakpoint of *e2820* corresponded precisely to the terminus of a small 12-bp hairpin that is diagnostic of the 3'-ends of Helitron transposons (ARKHIPOVA and MESELSON 2005), whereas one breakpoint of *e2825* was just upstream of this hairpin (Table 5). The *e2825* deletion breakpoint contained an insertion that was identical to the 3'-end of a homologous Helitron transposon and 121 bp of adjacent unique sequence found on cosmid C11G10, which is located 20 MU to the right of *unc-58* on chromosome X. Although the Helitron

TABLE 4
Breakpoints of *unc-58* deletions from a *clk-2(mn159)* background

<i>unc-58(e2827)</i>
CCGGAAAGATATCGAAAGGATCAGGATCCAAACCA..3993.. <u>gaatcttcgctcggtatgaagcaagttgcgtctg</u> agattccactaattattgttttttaatgtaaactg..25682. <u>GAAATGGTTTTTGTATCAATTGAAACAGTCTCCGT</u>
<i>unc-58(e2828)</i>
TTCGGGACACGATGGTGGAACTGGAGCTTCGAGTT..4523..ggactggtattaacacgcaggattgcatggga TTTTTAGT (insert) tagatagagcattccgtgat <u>aattttcattccggtg</u> ..39000. <u>AAATTTTCATTCCGTGATAATTTTCATTCCGTGA</u> (direct repeats)
<i>unc-58(e2829)</i>
GTGTGAACAAGAGTCTTCAAAATTCTGAATTATTC..13421.. <u>accagaataacttctcgagattcttcaatttatttt</u> acagaagaaacaggtgaagcacatagctccaagaa..14020.. <u>ACCGGTAGCGGTTGTGATCCGGTCCATAGTTCTT</u>
<i>unc-58(e2830)</i>
CTCGAACCCATTTTTTTGATGGGATACTGTAACCTT..12374.. <u>tgtgacatcaactctcacacagcccaccgccaatt</u> ATTCTAAAGTAGTGAATTCTGGTGAAAGTAGTGAATTCTGTGAATT (insert) accgaaaactttcaattaactaccgttttgctcga..10051.. <u>CAAAGTAGTGAATTCTGGTGAAGTACGCCTTGAG</u>
<i>unc-58(e2831)</i>
GTTGAATCAATAAACCAAAATAGGTACCTATTGGG..11302.. <u>aaccaaagtttctcttcagttccgtcgacaccaag</u> TGCTACT (insert) tcggcagctggtatgaacataagtggtataccaac..11520.. <u>AACGCGAATGCTACTGAGAAAACCTTTCCGACGTC</u>
<i>unc-58(e2832)</i>
AATAACTGGCAGGCAAGTTTTGTTTCCTTATTGT..9055.. <u>gaggctacatcttcaacagttctatgatttcaattg</u> caagaaccggttaggcggttgatccggtccata..14048. <u>GTTCATGATTCATCTATTATGAAACGGAGCTGT</u>

Sequence is annotated as for Table 1. Positions relative to the cosmid T06H11 are indicated.

transposons that lie within C11G10 and the *unc-58* locus share identical hairpin and 3'-end sequences, polymorphisms exist immediately 5' to the hairpin that suggest that the genesis of *e2825* involved a recombination event that occurred between 15 and 45 bp upstream from the 3' hairpin. Thus, five *unc-58* deletions that were identified in checkpoint-defective backgrounds contained either a polypurine tract or direct or inverted repeats at or near their breakpoints.

Given the unusual sequences observed at some deletion breakpoints, genome sequence corresponding to 35 bp upstream and downstream of each deletion breakpoint identified in this study was assessed for its propensity to form secondary structures using the Mfold program (ZUKER 2003). Of 44 sequences corresponding to 22 deletion breakpoints, 10 were predicted to form hairpins >8 bp in length. In comparison, of 68 contiguous 70-bp segments of sequence corresponding to the genomic DNA of the short *unc-58* transcript, 4 were predicted to fold into hairpins >8 bp ($P = 0.018$, Fisher exact test). Of the 20 *unc-58* breakpoints that fell within the genomic region of the short *unc-58* transcript, 7 could fold into hairpins >8 bp in length, which was also significantly different from the frequency observed for 68 contiguous segments of sequence from the same interval ($P = 0.002$, Fisher exact test). Thus, breakpoints of spontaneous mutations that occurred in

checkpoint-defective backgrounds were enriched for sequences that could form hairpins.

Sequencing of spontaneous *mrt-2*-induced visible mutations revealed that G-tracts flanked two of the four breakpoints (Table 1). Three G-tracts span the 5'-end of the *unc-58* gene, but no G-tract-induced deletions were recovered among 17 *unc-58* deletions examined, even though several of these deletion events removed one or more of the G-tracts (Figure 4A, Tables 2 and 3). Thus, the G-tracts adjacent to breakpoints of deletions that occurred in the *mrt-2* background may have been fortuitous, although G-tracts greater than nine nucleotides in length are rare in the *C. elegans* genome (Figures 1 and 4; S. AHMED, unpublished data). *C. elegans dog-1* (*deletion of guanine tract-1*) mutants display small deletions where a G-tract always flanks one of the breakpoints, and previous PCR-based analysis revealed that some, but not all, G-tracts >22 bp in length are capable of generating deletions (CHEUNG *et al.* 2002). Therefore, the G-tracts in the *unc-58* gene may be of the "stable" variety, and some caution may be warranted when assessing the relevance of the mutation spectra obtained at this locus.

To determine if *mrt-2* displays a general defect in G-tract stability, genomic DNA from *mrt-2* mutants was examined at two G-tract loci that were previously shown to be highly unstable in the *dog-1* background: F55F3

TABLE 5
Breakpoints of *unc-58* deletions from a *mrt-2* background

<i>unc-58(e2815)</i>
AACCTGACAATAATAAATTTTAATTATTTCTGAAT . . 11665 . . <u>ta</u> tgatcaacaataccgcaagtagttaacacagt cgaaggcaatggttgcgaaaacggcaagagtcagaa . . 13229 . . <u>TATTTT</u> GAGAGCGCTGATCATGCGTTCTTTTCTAA
<i>unc-58(e2816)</i>
ATTC <u>AAAAATTATAATGAAAGATAAAAGAGAAAAA</u> . . 9418 . . gttgttcgcatgagtcgcaacaagattgggcaaa (polypurine tract) TTT (insert) aaaccataaatt <u>tcgttcgttc</u> accattt <u>ggaac</u> tg . . 11192 . . TTAG <u>GATACGA</u> TATTTCAATTATGATTTAAATAA (hairpin)
<i>unc-58(e2817)</i>
CTGTTAGGATACGATATTTCAATTATGATTTAAAT . . 8919 . . gtaatttgttaccagctcttactatcaagtaggct atcgccccagtaaagcaacgatt <u>ttcctttgagt</u> tc . . 9976 . . CTC <u>ACTCA</u> TTCC <u>AAGAA</u> TCTGTAAACATTCGAAAT (hairpin)
<i>unc-58(e2818)</i>
TCCTTACCGATCGGAAACCATAATTT <u>TCGTTTCGTTTC</u> . . 11177 . . accattt <u>ggaact</u> gttag <u>gatacga</u> tatttcaatt (hairpin) taattcaggtaaaagaatgttactataagatgttt . . 11996 . . CCCTGAAAGAAATCAAACACACAGTTACATTATAA
<i>unc-58(e2819)</i>
ATTCGCGCTCCATCATTAAGAACAACCTTTGATA . . -2818 . . <u>ata</u> <u>tca</u> <u>aga</u> <u>ca</u> cgatgatgcgatggcaaacat T (insert) ctacagtaccccgactatatccctacactaagccc . . 15255 . . <u>AAC</u> <u>TCACAT</u> <u>TCC</u> <u>TCC</u> GAGAAGCCAAAACCTTCATAG
<i>unc-58(e2820)</i>
AAACTTTC <u>AAATTAAC</u> <u>TACCGTTTTG</u> CTCGAC <u>CAAA</u> . . 10055 . . <u>gtagtgaatt</u> ctggtgaagtacgccttgagatgtg (hairpin) athtagatgcgtaaaactaatataagtaaccagcc . . 15117 . . <u>GTGGACCGCACC</u> TCC <u>GGCGCGGCC</u> CGACTTCTGG (hairpin)
<i>unc-58(e2821)</i>
TAGAATTACATACTAGTTACATAACCTTTGCGGCC . . 9791 . . ccatacagtaaaaacgtaccagaagatgtttcgtca T (insert) gtgttattatcttatcaaaactcactgctccttg . . 42818 . . ATATATATTTCCAGCCAGCGAACTCCACCATT
<i>unc-58(e2822)</i>
AAAGCAG <u>GTTTTTGTGTTCC</u> CGTTTTCAAGTTGT . . 9378 . . <u>cagaattcaaaaat</u> tataatgaaagataaaagag (hairpin) gacagaaacggaaaaaactttaataaaacagcaa . . 10966 . . GATAGATGACATTGATAGACAAGAGTTGTATGTTA
<i>unc-58(e2824)</i>
TGGGAAGGACTTGACCCAGCCAACAAGAAGTTAT . . 7656 . . cattctgggagggcggagacacggctactgactgta ataaatattattgt <u>gaaaaaaaaatcaaa</u> atgtgca . . 34517 . . <u>GAAAAAATGCTCAAA</u> GAAACCTTTGTTTAAAGAT (direct repeat)
<i>unc-58(e2825)</i>
TATGTGTGAACAAGAGTCTTCAAAATTCGAATTA . . 13421 . . <u>ccc</u> accagaatacttctcgagattcttcaatttat <u>TCCAATGAACCAAAATTTGTAGAGTATTTTGCTAAAGATAGTCTAACTGGAATTCGATCTGCTATTA</u> AAAAACCAAAAAATAGAAACA AAACAGAAATGGTCCCATGGAATCCAAATATTA AAAAACAGC <u>CGTGGACCGCACC</u> TCC <u>GGCGCGGCCCGCG</u> ACTTCTGAGGCTGA AAACTAATTTTCTGAAACTACCGTAA (insert) (hairpin) gggggtgaaaactaattttctgaaactaccgtaa . . 15109 . . TCATACAGCACTCCTACCGTAACCTATTGTACCA
<i>unc-58(e2826)</i>
TTAAATCTTATTTTAAGCCTTGACAATCTGAATAT . . 10280 . . <u>AAAt</u> <u>aaact</u> ctaaaatattgcacaaatatttgccg gatgacattgatagacaagag <u>gttgatggtt</u> aattg . . 11005 . . <u>aaaCAAAACAAC</u> ACTCAAAGAAGACATAATCATGT (hairpin)

Sequence is annotated as for Tables 1 and 4, except for the stretch of *e2825* breakpoint sequence whose origin is ambiguous as a consequence of a recombination event, which is in italics.

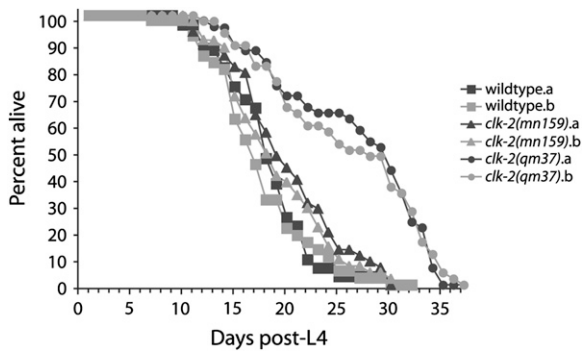


FIGURE 5.—*clk-2(qm37)* but not *clk-2(mn159)* is long lived. Life spans of strains that were independently crossed nine times *vs.* a *dpy-17;unc-32* marker strain ($n = 100$ nematodes scored for each strain).

(tract length of C_{26}) and F38A6 (tract length of C_{29}). In addition, a third locus, Y41E3 (tract length of C_{17}), for which no deletions were previously observed, was examined. PCR-based deletion products were not observed for any of these loci using either *mrt-2* or *dog-1* genomic DNA as a template ($n = 30$ for each genotype) (data not shown), despite extensive optimization of PCR conditions and robust wild-type-sized PCR products. Thus, this PCR-based assay may vary from lab to lab (M. TIJSTERMAN and R. PLASTERK, personal communication), and we were unable to confirm that the *mrt-2* mutant has a general defect in G-tract stability using this PCR-based approach.

***clk-2(qm37)* but not *clk-2(mn159)* has an extended life span:** *unc-58(e665)* Mutator assays suggested that *clk-2(mn159)* had a deletion-based genome stability defect whereas *clk-2(qm37)* did not (Figure 3A). Interestingly, *clk-2(qm37)* has checkpoint defects that are modestly more severe than those of *clk-2(mn159)* (Figure 2), and the *clk-2(qm37)* mutant has a stronger Slow Growth phenotype than *clk-2(mn159)* (AHMED *et al.* 2001). Given the phenotypic disparity between the *clk-2* alleles, and given that *clk-2(qm37)* has been reported to confer an extended life span (LAKOWSKI and HEKIMI 1996), we sought to determine how strains of *clk-2(qm37)* and *clk-2(mn159)* compared with respect to their effects on longevity. Life spans of independently outcrossed *clk-2(qm37)* strains ($a = 30.4 \pm 1.4$ days; $b = 29.4 \pm 1.6$ days) were significantly greater than either *clk-2(mn159)* ($a = 21.1 \pm 0.8$ days; $b = 20.3 \pm 0.8$ days) or N2 wild type ($a = 20.7 \pm 0.9$ days; $b = 21.4 \pm 1.0$ days) strains ($P < 0.002$ in all cases; log-rank Mantel Cox test), whereas *clk-2(mn159)* strains were not significantly longer lived than wild-type strains ($P = 0.67, 0.84, 0.62,$ and 0.21 for comparisons with a and b wild-type strains, respectively), revealing a fourth difference between these two *clk-2* alleles (Figure 5). Thus, the *clk-2* mutations *qm37* and *mn159* have distinct properties with respect to all phenotypes examined, as might be expected for hypomorphic alleles of an essential gene.

DISCUSSION

Our results indicate that DNA damage response genes are required to suppress the frequency of spontaneous mutation in *C. elegans*. Of six spontaneous mutations isolated from the *mrt-2* background and one spontaneous mutation isolated from the *clk-2(mn159)* background, all but one occurred as a consequence of deletions (Figure 1, Table 1). In addition, 26/36 *unc-58* mutations identified in *mrt-2*, *hus-1*, or *clk-2(mn159)* genetic backgrounds were deletions, whereas no deletions were observed for 11 *unc-58* mutations in wild-type or *clk-2(qm37)* backgrounds. In agreement with these observations, a previous study revealed that 11% of spontaneous mutations in the *C. elegans unc-54* gene were found to be deletions, indicating that deletions are rare events (PULAK and ANDERSON 1988). Similarly, the frequency of spontaneous deletions segregating in human families corresponds to 5–10% of all mutations (VERHOOG *et al.* 1998; PUGET *et al.* 1999; COOPER 2002).

A recent study in *Schizosaccharomyces pombe* indicated that the frequency of spontaneous deletions that occurred as a consequence of DNA polymerase α -mediated replication stress were suppressed by hypomorphic alleles of *rad17* (KAI and WANG 2003). These results are surprising as *rad17* encodes a component of the replication factor C complex that loads the 9-1-1 complex onto DNA damage, so mutation of 9-1-1 complex subunits in *C. elegans* might be expected to suppress rather than promote the formation of spontaneous deletions. Note, however, that deficiency for Cds1/Chk2, an effector kinase that acts downstream of the 9-1-1 complex, dramatically enhanced the frequency of deletions, but not of point mutations, in response to DNA polymerase α -mediated replication stress (KAI and WANG 2003). Therefore, the deletions observed in our study may ultimately reflect malfunction of downstream signaling components of the DDR pathway such as Chk1 or Chk2.

A genomewide RNA interference (RNAi) screen recently identified *mrt-2* as a gene that may suppress small deletions or insertions of one or more nucleotides in a repetitive array of an out-of-frame LacZ reporter construct in a manner that is independent of mismatch repair (POTHOF *et al.* 2003). However, the majority of mutations identified in the checkpoint-defective backgrounds used in our study were spontaneous deletions large enough to be detected by agarose gel electrophoresis. The other mutations may have been point or frameshift mutations. The *dpy-31* mutation isolated from the *mrt-2* background resulted from a T-to-C point mutation (NOVELLI *et al.* 2004), and two additional *mrt-2*-induced spontaneous mutations at the *unc-93* locus arose as a consequence of a T-to-C transition and a T-to-G transversion (data not shown). Thus, limited sequence analysis failed to reveal frameshifts arising from the *mrt-2* background. The restoration of

reading frame by *mrt-2*(RNAi) may therefore have occurred as a consequence of larger deletions within the repetitive LacZ array. However, a more thorough examination of point or frameshift mutations from *mrt-2* or other checkpoint-defective backgrounds may reveal increases in the frequency of frameshifting. Note that deficiency for the 9-1-1 complex or downstream mediator checkpoint kinases such as Chk1/Chk2 has been shown to affect the frequency of frameshifting in response to replication stress in *S. pombe* (KAI and WANG 2003).

Although both *clk-2(mn159)* and *clk-2(qm37)* have strong defects for the S-phase DNA replication checkpoint that responds to stalled replication forks (AHMED *et al.* 2001), only *clk-2(mn159)* displayed a Mutator phenotype (Figure 3A). This result was surprising because S-phase checkpoint defects in *Saccharomyces cerevisiae* typically confer high frequencies of spontaneous GCRs relative to defects in the 9-1-1 complex (MYUNG *et al.* 2001a,b). The pleiotropic nature of *clk-2* mutations extends to additional phenotypes, such as growth rate and life span, where only the *qm37* allele has pronounced effects (Figure 5) (LAKOWSKI and HEKIMI 1996; AHMED *et al.* 2001). The life span of *clk-2(mn159)* was not significantly different from that of wild type (Figure 5), indicating that neither a strong S-phase checkpoint defect (AHMED *et al.* 2001) nor an enhanced frequency of endogenous mutation is sufficient to affect *C. elegans* life span. Note, however, that deficiency for several additional S-phase checkpoint genes has recently been shown to confer an extended life span in *C. elegans* (OLSEN *et al.* 2006) and that the differential effects of the *mn159* and *qm37* alleles of *clk-2* on longevity may provide an opportunity to more precisely determine the relationship between the S-phase checkpoint and life span.

Frequencies of spontaneous mutation were 8- to 15-fold higher for *hus-1* and *mrt-2* mutants than for wild type (Figure 3). These mutation frequencies mirror the 7- to 10-fold increases in GCRs observed for *S. cerevisiae rad9* and *rad17* mutants (homologs of *hus-1* and *mrt-2*, respectively) and for mutations that affect the *S. cerevisiae rad51* homologous recombination (HR) pathway (MYUNG *et al.* 2001b). If endogenous DSBs are not accurately repaired by checkpoint-orchestrated HR, which usually relies on a sister chromatid for repair, then resection and end-joining of DSBs may occur (YU and GABRIEL 2003), as observed for many mutations that occurred in checkpoint-defective backgrounds in this study. One *unc-58* deletion breakpoint, *e2825*, occurred near the 3'-end of a nonautonomous Helitron transposable element and was unique because sequence from a homologous repetitive element elsewhere on chromosome X was inserted at the *e2825* breakpoint (Table 5). Thus, the *e2825* deletion may have been created by a break in the Helitron transposable element at the *unc-58* locus, strand invasion of a homologous transposon on

C11G10, and dissociation and end-joining at the *unc-58* locus to seal the break. These results suggest that *C. elegans* checkpoint-defective mutants may be deficient for a model of HR-dependent break repair known as synthesis-dependent strand annealing, which may involve processive cycles of template-directed DNA synthesis followed by dissociation to search for homology with the other end of the break (NASSIF *et al.* 1994; McVEY *et al.* 2004a). Recombination events similar to that of the *e2825* deletion have been observed for DNA sequences flanking *P* elements in *Drosophila* (Tsubota and HUONG 1991; HESLIP *et al.* 1992). In addition, studies in *S. cerevisiae* have indicated that DSBs in Ty retrotransposons can result in gene conversion (PARKET *et al.* 1995) and translocations (LEMOINE *et al.* 2005), and recombination between Ty elements represents a major source of genome rearrangements (DUNHAM *et al.* 2002). Roughly 2% of the *C. elegans* genome is composed of autonomous and nonautonomous Helitron elements (KAPITONOV and JURKA 2001), although their present activity is currently uncertain.

For two of five spontaneous deletions that gave rise to visible mutant phenotypes in the *mrt-2* background, a G-tract was present immediately adjacent to one of the breakpoints. The DOG-1 helicase has been shown to suppress deletions at such G-tracts, and one model suggests that this helicase may resolve unusual non-Watson-Crick structures formed by G-tracts during replication of the lagging DNA strand prior to DSB induction (CHEUNG *et al.* 2002). Given that similar deletions were recovered from a *mrt-2* background (Table 1), the MRT-2 protein may help to recruit DOG-1 to resolve unusual G-rich structures prior to DSB induction. Alternatively, both *mrt-2* and *dog-1* may act downstream of DSB induction to facilitate synthesis-dependent strand-annealing-mediated DSB repair. Note that the mammalian DOG-1 homolog BRIP1/BACH1 has been implicated in the response to interstrand crosslinks that are processed via a DSB intermediate (MAGANA-SCHWENCKE *et al.* 1982; BRIDGE *et al.* 2005). The sizes of the deletion events observed in the absence of *dog-1* were only several hundred base pairs in length (CHEUNG *et al.* 2002), whereas the events observed at *mrt-2* G-tract deletions were several kilobases and most deletions in the *mrt-2*, *hus-1*, and *clk-2(qm37)* backgrounds were >300 bp. Thus, it is possible that the DDR mutants used in this study have an additional defect that renders DSBs more susceptible to exonuclease-mediated degradation prior to end-joining. Alternatively, the initial visible mutants isolated from the *dog-1* background may have represented the short end of the *dog-1* deletion spectrum (CHEUNG *et al.* 2002). In summary, our genetic results suggest that *dog-1* and *mrt-2* may act to repress genome instability caused by G-rich non-Watson-Crick DNA structures such as G-quadruplexes.

Several *unc-58* deletions had unusual sequences at or near their breakpoints, including short direct repeats,

short hairpins, and long G/A-rich polypurine tracts. Thus, the initiation of a spontaneous deletion event may involve stretches of nucleotides with propensities to form slipped intermediates, which may be vulnerable to endonucleolytic attack and result in replication fork stalling and collapse. Stalled replication forks have been shown to result in DSB formation in *Escherichia coli* (MICHEL *et al.* 1997), which may lead to exonuclease-mediated DSB resection and end-joining if checkpoint-mediated homologous recombination fails to resolve the lesion (MOORE and HABER 1996; YU and GABRIEL 2003; GUIROUILH-BARBAT *et al.* 2004). Note that polypurine tracts and direct or inverted repeats have been observed at genome rearrangement breakpoints in bacteria, yeast, and humans (ABEYSINGHE *et al.* 2003; CHUZHANOVA *et al.* 2003; BACOLLA *et al.* 2004). The biological relevance of such structures *in vivo* is emphasized by the two *unc-58* deletions with breakpoints at or near a stem loop at the 3'-end of a Helitron transposable element. This 3' stem loop is conserved in other Helitron transposons, is essential for transposition, and may facilitate termination of rolling circle replication by direct inhibition of the DNA replication machinery (MENDIOLA *et al.* 1994; KAPITONOV and JURKA 2001; TON-HOANG *et al.* 2005). This suggests a model where the Helitron stem loop may be sufficient to induce spontaneous DSBs at a low frequency. As hairpins were significantly enriched for near deletion breakpoints, such sequences may be relevant to the genesis of DSBs or may aid in sealing resected breaks. Although some deletion breakpoints that we identified were close to short direct repeats, short hairpins, and long G/A-rich polypurine tracts, potential roles for these sequences in the genesis of chromosome rearrangements in *C. elegans* may be bolstered by analysis of many more spontaneous *unc-58* deletion events or by transgene reporter assays (POTHOF *et al.* 2003).

Our data suggest that DNA damage response proteins help to maintain genome stability by coordinating proper HR-mediated repair of spontaneous DSBs, some of which are likely to occur as a consequence of unusual nucleic acid structures that form during DNA replication (BACOLLA *et al.* 2004; LOVETT 2004). Defects in DNA damage response proteins such as ATM, ATR, BRCA1, BRCA2, MRE11, and NBS1 result in familial cancer syndromes and confer GCRs such as inversions, translocations, or large deletions suggestive of DSB repair defects (VENKITARAMAN 2002). A recent study of BRCA1 and BRCA2 tumors revealed a number of large hemizygous deletions based on comparative genome hybridization using microarrays with an average resolution of 1 Mb (JONSSON *et al.* 2005). Further, the second allele of BRCA1 or BRCA2 is almost always inactivated by genomic rearrangements or deletions in cancers that develop in patients who are heterozygous at one of these loci (WELCSH and KING 2001; HENDRICKSON *et al.* 2005). Although we recovered

one visible mutant from a *mrt-2* background with an ~230-kb deletion, *mrt-2* and *clk-2(mn159)* defects resulted in 22 additional spontaneous deletions that ranged from 0.15 to 35 kb. Note that the *unc-58* target gene is located on the X chromosome, which harbors few essential genes, and that the closest genes annotated to possess sterile or lethal phenotypes were located 71 kb to the left of and 101 kb to the right of *unc-58* (<http://www.wormbase.org>). Thus, small- to medium-sized deletions occur more frequently than large chromosomal aberrations in checkpoint-defective *C. elegans* mutants. Gross changes to chromosomes that are visible by microscopy and microsatellite analyses may contribute to the development of cancer. However, our results suggest that genomes of cancer cells that evolve in backgrounds with DNA damage response defects may be peppered with small- to medium-sized deletions and that the resulting aberrant gene products or hemizygosity may be a driving force in the development of cancer.

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