

Developmental Modulation of Nonhomologous End Joining in *Caenorhabditis elegans*

Iuval Clejan,^{*,†} Julie Boerckel^{*,‡} and Shawn Ahmed^{*,†,‡,1}

^{*}Department of Genetics, [†]Lineberger Comprehensive Cancer Center, [‡]Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599-3280

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ABSTRACT

Homologous recombination and nonhomologous end joining (NHEJ) are important DNA double-strand break repair pathways in many organisms. *C. elegans* strains harboring mutations in the *cku-70*, *cku-80*, or *lig-4* NHEJ genes displayed multiple developmental abnormalities in response to radiation-induced DNA damage in noncycling somatic cells. These phenotypes did not result from S-phase, DNA damage, or mitotic checkpoints, apoptosis, or stress response pathways that regulate dauer formation. However, an additional defect in *him-10*, a kinetochore component, synergized with NHEJ mutations for the radiation-induced developmental phenotypes, suggesting that they may be triggered by mis-segregation of chromosome fragments. Although NHEJ was an important DNA repair pathway for noncycling somatic cells in *C. elegans*, homologous recombination was used to repair radiation-induced DNA damage in cycling somatic cells and in germ cells at all times. Noncycling germ cells that depended on homologous recombination underwent cell cycle arrest in G₂, whereas noncycling somatic cells that depended on NHEJ arrested in G₁, suggesting that cell cycle phase may modulate DNA repair during development. We conclude that error-prone NHEJ plays little or no role in DNA repair in *C. elegans* germ cells, possibly ensuring homology-based double-strand break repair and transmission of a stable genome from one generation to the next.

TWO main pathways exist for the repair of radiation-induced DNA double-strand breaks (DSBs): homologous recombination (HR) and nonhomologous end joining (NHEJ; LIEBER *et al.* 2003). HR utilizes regions of sequence identity on a sister chromatid or a homologous chromosome to initiate strand invasion, which can provide error-free repair. In contrast, NHEJ joins broken DNA ends in the absence of substantial homology and may result in errors such as nucleotide addition or removal, depending on the nature of the damaged ends and how they are processed prior to ligation. Key proteins that participate in canonical NHEJ have been identified, including the Ku70/Ku80 DSB-binding heterodimer that functions by protecting broken DNA ends from degradation and by recruiting DNA ligase IV and its cofactor XRCC4 to seal the break (LIEBER *et al.* 2003). The Mre11p/Rad50p/Xrs2p (MRX) complex is required for NHEJ only in budding yeast (MOORE and HABER 1996; HARFST *et al.* 2000; YEO *et al.* 2000; MANOLIS *et al.* 2001; HUANG and DYNAN 2002), where it may mediate nucleolytic processing of DSBs. In addition, the MRX complex participates in HR in all organisms examined (BENDER *et al.* 2002; STRACKER

et al. 2004). Two proteins required for NHEJ in vertebrates are the DNA-dependent protein kinase catalytic subunit that interacts with Ku70/Ku80 (HARTLEY *et al.* 1995) and the Artemis nuclease that can process hairpins and perhaps other structures that occur at DSBs (MA *et al.* 2002). In yeast, *Drosophila*, *Arabidopsis*, and vertebrates, end joining of DSBs can occur in the absence of canonical NHEJ via a pathway that relies on patches of microhomology at DNA ends to join DSBs (FELDMANN *et al.* 2000; MA *et al.* 2003; YU and GABRIEL 2003; HEACOCK *et al.* 2004; McVEY *et al.* 2004). The yeast microhomology end-joining pathway is partially dependent on DNA ligase IV and requires the MRX nuclease complex and the Rad1/Rad10 heterodimer that cleaves 5' or 3' flaps (MA *et al.* 2003; YU and GABRIEL 2003).

NHEJ has been reported to occur cooperatively (MILLS *et al.* 2004; RAPP and GREULICH 2004), competitively (ADACHI *et al.* 2001; ALLEN *et al.* 2003; SCHAR *et al.* 2004), or as a backup to HR (BOULTON and JACKSON 1996; SIEDE *et al.* 1996). In budding and fission yeast, HR is the primary means of DSB repair in proliferating haploid cells, whereas NHEJ is utilized for DSB repair during G₁ or G₀ phases of the cell cycle (SIEDE *et al.* 1996; KARATHANASIS and WILSON 2002; FERREIRA and COOPER 2004). In vertebrate cells in culture, NHEJ can occur at all stages of the cell cycle, whereas HR is restricted to the

¹Corresponding author: Department of Genetics, Coker Hall, University of North Carolina, Chapel Hill, NC 27599-3280.
E-mail: shawn@med.unc.edu

S-phase and G₂-phase (TAKATA *et al.* 1998; ROTHKAMM *et al.* 2003; SALEH-GOHARI and HELLEDAY 2004; HINZ *et al.* 2005). Deficiency for NHEJ causes more severe DSB repair defects in vertebrate cells than in yeast (PASTINK *et al.* 2001). The endogenous levels of these repair processes may reflect the prevalence of repetitive DNA sequences, which are abundant in vertebrate genomes and may preclude use of a homologous chromosome for error-free repair at most stages of the cell cycle (TAKATA *et al.* 1998).

Although DNA damage response defects play an important role in the development of cancer, little is known about differential utilization of DNA repair pathways in multicellular organisms *in vivo*. In this work, we characterize the relative importance of HR and NHEJ in the *Caenorhabditis elegans* germ and somatic cells at multiple developmental stages. Canonical NHEJ appears to be used exclusively by nondividing somatic cells, whereas HR is used to repair radiation-induced DNA damage in proliferating somatic cells and in germ cells at all times. Noncycling germ cells may ensure accurate DSB repair by arresting at a cell cycle stage where template-dependent DSB repair can be achieved using a sister chromatid.

MATERIALS AND METHODS

Strains: *C. elegans* strains were cultured at 20° as described (SULSTON and HODGKIN 1988), unless otherwise indicated. All NHEJ mutant strains were outcrossed to the *dpy-17unc-52* double mutant at least six times prior to analysis. The following strains were used: RB964 *cku-80(ok861)*, YA942 *cku-80(tm1203)*, RB873 *lig-4(ok716)*, YA679 *lig-4(tm750)*, YA982 *cku-70(tm1524)*, DR26 *daf-16(m26)*, DR85 *daf-8(m85)*, DR1564 *daf-2(m41)*, DR1572 *daf-2(e1368)*, SP506 *rad-5/clk-2(mn159)*, WS2277 *hus-1(op241)*, TG9 *dpy-13(e184)*, *rad-51(lg8701) IV/nT1[let-?(m435)] (IV;V)*, AV112 *mre-11(ok179)/nT1*, CB164 *dpy-17(e164)*, CB189 *unc-32(e189)*, CB47 *unc-11(e47)*, BC119 *dpy-24(s71)*, SP506 *rad-5/clk-2(mn159)*, VC172 *cep-1(gk138)*, CB1511 *him-10(e1511)*, MT1522 *ced-3(n717)*, NL2099 *rrf-3(pk-1426)*, and MD1971 *egl-8(md1971)*. Quantitative data for figures and tables in this article were typically obtained using the reference alleles *cku-80(tm1203)* and *lig-4(ok716)*.

Egg Rad assays: Up to 20 well-fed young adults were allowed to lay eggs for 1–2 hr. Staged embryos either were irradiated within 15 min (early stage embryos) or allowed to develop for 3–4 hr prior to irradiation (late-stage embryos). Each time a new strain was used, synchrony of late-stage embryos at approximately the bean stage was verified by Normarksi microscopy. Gamma irradiation was performed in a Shepherd Mark IV Cs137 irradiator at a dose rate of 430 rad/min. The Slow Growth (Gro) phenotype was scored 48 hr after irradiation by comparing unirradiated controls with irradiated experimental animals, whereas other phenotypes were scored after 96 hr, unless otherwise indicated. The Protruding Vulva (Pvl) and Ruptured (Rup) phenotypes were scored using a dissection microscope. To score the Egg-Laying Defective (Egl) phenotype, worms were singled and those that did not lay eggs 4 days after irradiation were examined using Normarksi microscopy for the presence of advanced-stage eggs in their uteri. For the Vulvaless (Vul) phenotype, the lack of a vulva or an incomplete opening was scored using Normarksi micros-

copy. The Uncoordinated phenotype (Unc) was scored on the basis of sluggish movement and failure or sluggishness in response to touch with a pick either on the head or on the tail (usually both). Dauer-constitutive mutants were raised at the restrictive temperature of 25° to obtain dauers, which were then irradiated and left at 25° for 1 day, before downshifting to 20° to allow for dauer recovery. For experiments involving *him-10*, strains were shifted to 25° after irradiation, the Slow Growth phenotype was scored 40 hr later, and all other phenotypes were scored 72 hr later.

Genetics: The presence of homozygous NHEJ mutations was assessed by scoring for Late Egg Rad phenotypes. For *hus-1* doubles, doubles of *unc-11* with *cku-80* or *lig-4* were first constructed, crossed with *hus-1*, and the presence of *hus-1* in non-Unc, Late Egg Rad F₂ homozygotes was confirmed by irradiating F₄ L1's at 60 Gy and scoring for almost complete sterility. For *daf-16* doubles, *dpy-24* doubles with *cku-80* or *lig-4* were constructed, crossed with *daf-16*, and Late Egg Rad F₂ plates that were non-Dpy/+ were allowed to starve and scored for few survivors after 1 month to confirm the presence of *daf-16*. For *daf-12* doubles, *cku-80* or *lig-4* males were crossed with *daf-12*, F₁ males were backcrossed with *daf-12*, and F₂ progeny from backcrossed F₁ were scored for Late Egg Rad phenotypes. For doubles with *daf-2*, a *daf-2;dpy-17* double was constructed, crossed with *cku-80*, and non-Dpy, dauer recombinants were picked. For *rad-5/clk-2;cku-80* doubles, *unc-32;cku-80* and *rad-5/clk-2;dpy-17* doubles were first constructed, *trans*-heterozygotes of these doubles were generated, non-Unc/+ F₃'s were isolated from recombinant non-Dpy/+ F₂'s, and the *cku-80* genotype was confirmed by the Late Egg Rad phenotype, whereas the *rad-5/clk-2* genotype was confirmed by embryonic lethality at 25°. For construction of the *cku-80;lig-4* doubles, markers for the *rad-5/clk-2;cku-80* doubles above were used, and recombinant genotypes were confirmed by PCR for both deletions.

Suppression assays: For each double mutant, at least four independent lines were obtained that were tested for radiation-induced Gro and Unc phenotypes using the Late Egg Rad assay. For each line, at least 50 embryos were irradiated.

Microscopy: A Nikon E800 fluorescence microscope was used with either a green fluorescent protein (GFP) or 4',6-diamidino-2-phenylindole (DAPI) filter set. For GFP experiments, worms were placed on slides with an agarose pad and sealed with microscope oil around the coverslip. For DAPI experiments, worms were fixed in 200 ng/ml DAPI in 95% ethanol, rehydrated in M9, and placed on a slide with a drop of NPG (3.6% weight/volume *n*-propylgallate, 140 mM Tris base, 90% volume/volume glycerol). Pictures were taken with a Hammamatsu Orca ER camera.

Immunofluorescence: Immunostaining was performed as previously described (KAWASAKI *et al.* 1998). Briefly, embryos were freeze cracked, fixed in cold methanol, and stained with mouse anti-P granule antibody OIC1D4 (1:3; Developmental Studies Hybridoma Bank, Iowa City, IA) or with rabbit antiphospho-histone H3 primary antibody (1:200; Upstate, Charlottesville, VA), using fluorescein isothiocyanate-conjugated goat anti-mouse and rhodamine-conjugated goat anti-rabbit secondary antibodies, respectively (1:150; Jackson Immuno-Research Laboratories, West Grove, PA).

DAPI staining and microscopy of dauer germlines: *daf-2L4* hermaphrodites were placed at 25° for 4 days, their progeny were moved by mouth pipette to a water glass and simultaneously fixed and stained in a 200 ng/ml DAPI/ethanol solution, allowed to rehydrate overnight in M9 buffer at 4°, and then mounted in NPG. Germ-cell nuclei were easily visible as a closely packed cluster of large nuclei on the ventral side of larval midsections. Z-stacks with 0.75-µm spacing were obtained using a Zeiss LSM 510 laser scanning confocal

microscope, and well-isolated nuclei were analyzed using Metamorph software. For each plane, an oval region was drawn manually around the cell of interest, the image was thresholded, and the gray-scale value was integrated for all the pixels above the threshold and within the oval. The threshold level was determined empirically to be as low as possible so that the visible nucleus would be included and so that changing the threshold value would not affect the integrated intensity by >5%. All cells were measured with the same threshold value. Background gray value was determined by drawing ovals roughly half the size of nuclei in at least two well-separated regions in each cell and by determining the average intensity with the threshold set to zero. Background was then subtracted for each nucleus by multiplying the average gray value by the number of pixels in the thresholded nucleus. For calibration, each intensity value thus obtained was normalized by the average intensity of at least two ventral nerve cord nuclei from the same stack and positive controls were intestinal nuclei. Two stacks were obtained for four worms and 24 germ nuclei were analyzed.

RNA interference: RNA interference (RNAi) by feeding was performed as previously described (KAMATH *et al.* 2001). Briefly, RNAi bacteria were streaked onto 12 µg/ml tetracycline, 50 µg/ml ampicillin LB plates from -80° stocks and grown overnight at 37°. A single colony was picked and grown in a 12 µg/ml tetracycline, 50 µg/ml ampicillin LB liquid culture for 6–8 hr, seeded onto NGM plates (50 µg/ml ampicillin, 12 µg/ml tetracycline, 2 mM isopropyl-β-D-thiogalactopyranoside), and grown at room temperature for 2 days. L4 larvae were placed on seeded RNAi plates, moved to new plates 24 hr later, and either F₁ or F₂ progeny were irradiated.

RESULTS

***C. elegans* NHEJ mutants:** Ku70, Ku80, and DNA ligase IV are core components of the canonical NHEJ machinery. The *C. elegans* orthologs of these proteins, CKU-70, CKU-80, and LIG-4, have been identified, and CKU-70 and CKU-80 have been shown to physically interact, as might be expected for subunits of the Ku heterodimer (BOULTON *et al.* 2002). *C. elegans* strains harboring deletions in the *cku-70*, *cku-80*, and *lig-4* genes were identified by groups screening genomic DNA derived from pools of mutagenized worms. From the published sequences (<http://www.wormbase.org/>), *cku-70(tm1524)* is an out-of-frame deletion plus insertion that removes part of the β-barrel DNA-binding domain and is predicted to result in a premature stop codon (Figure 1A). The *cku-80(ok861)* deletion results in a truncated in-frame transcript whose protein product is predicted to be missing part of the conserved α/β domain that is responsible for heterodimerization with the CKU-70 protein as well as the entire β-barrel domain that is responsible for DNA binding (Figure 1B). *cku-80(tm1203)* is also an in-frame deletion, predicted to be missing part of the α/β domain. *lig-4(ok716)* is an out-of-frame deletion that removes part of the catalytic ligase domain and is predicted to result in a premature stop codon. *lig-4(tm750)* is an in-frame deletion, whose protein product is predicted to be missing part of the catalytic domain and part of the DNA-binding domain

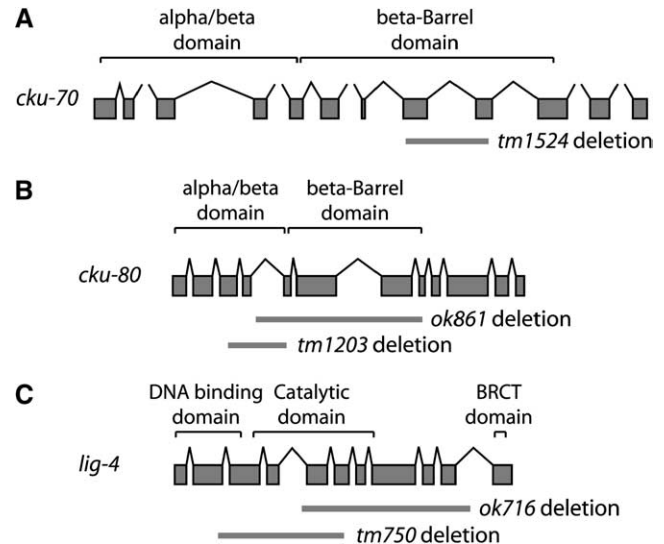


FIGURE 1.—Deletions of *cku-70*, *cku-80*, and *lig-4* remove essential regions of these NHEJ proteins. Gene structures are shown for (A) *cku-70*, (B) *cku-80*, and (C) *lig-4* (NCBI accession nos. CAB55094, CAA83623, and AAK85439, respectively). Predicted protein structural domains are shown above each gene model. Shaded bars below each gene model indicate positions of deletion mutations. Introns >1000 bp are not connected.

(Figure 1C). All of the above deletions are expected to produce strong loss-of-function phenotypes.

HR is the main form of DNA DSB repair in the *C. elegans* germline: As NHEJ defects in other organisms confer hypersensitivity to ionizing radiation (IR), strains deficient for *C. elegans* NHEJ genes were examined for this property. When L4 larvae (Figure 2A) of five different NHEJ mutant strains were irradiated with IR, progeny from their germlines did not display reduced levels of survival in comparison with wild type, with the exception of the *cku-70* strain, which displayed modestly enhanced radiation hypersensitivity at 120 Gy ($P = 0.003$) but not at 60 Gy ($P = 0.95$) (Figure 3A) (see additional *cku-70* results below). In contrast, progeny from mutants defective for *mrt-2*, a member of the 9-1-1 checkpoint heterotrimer that is loaded onto DSBs, displayed high levels of embryonic lethality relative to wild-type or NHEJ mutants at all IR doses (AHMED and HODGKIN 2000). Consistent with these observations, microscopy of oocyte nuclei of adults derived from L4 larvae irradiated with 120 Gy revealed that N2 wild-type or *cku-80* strains always displayed a normal complement of six paired bivalents, whereas *mrt-2* mutants displayed fragmented chromosomes in most nuclei (Figure 3B), indicating that *mrt-2* is critical for repair of IR-induced DSBs.

In *Saccharomyces cerevisiae*, HR is the main form of DSB repair, but a role for NHEJ is apparent in the absence of HR (BOULTON and JACKSON 1996; SIEDE *et al.* 1996). To assess if this might be the case in *C. elegans*, double mutants were constructed that were defective for both

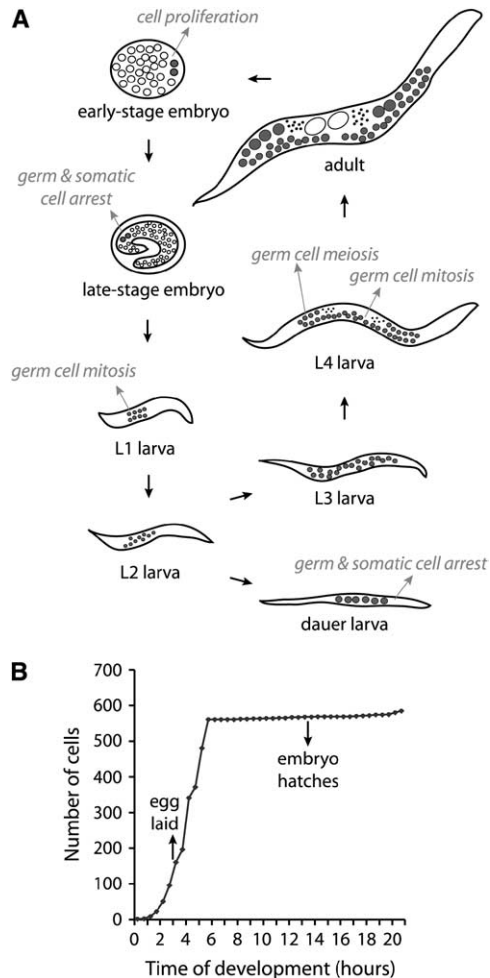


FIGURE 2.—Stages of *C. elegans* development used in this study. (A) Embryonic and larval stages (not to scale). Germ or somatic cells are depicted in shaded or open circles, respectively, sperm are tiny solid spots, and embryos are open ovals. (B) Cell proliferation occurs in early- but not late-stage *C. elegans* embryos.

NHEJ and *hus-1*, another member of the 9-1-1 checkpoint complex that responds to DSBs and participates in a signaling pathway that interfaces with HR-mediated DSB repair (SANCAR *et al.* 2004). *hus-1* mutants are only partially defective for HR-mediated DSB repair and display normal levels of fertility in the absence of exogenous DNA damage (AHMED *et al.* 2001; HOFMANN *et al.* 2002), allowing for analysis of the survival of progeny derived from irradiated germlines. This would not be possible if HR were completely lacking, which results in high levels of embryonic lethality, even in the absence of IR (CHIN and VILLENEUVE 2001; RINALDO *et al.* 2002; ALPI *et al.* 2003). Germlines of *hus-1* single mutants were hypersensitive to gamma irradiation (GARTNER *et al.* 2000; HOFMANN *et al.* 2002), but an additional defect in NHEJ failed to significantly enhance this phenotype (Figure 3C). Thus, NHEJ may be actively repressed in the L4/adult *C. elegans* germline,

even in the absence of proper checkpoint control, which allows for optimal HR to occur. The lack of hypersensitivity to IR in germlines of NHEJ mutant L4 larvae was not due to increased elimination of meiotic germ cells by apoptosis, because *hus-1* mutants are defective for IR-induced apoptosis in the germline (GARTNER *et al.* 2000; HOFMANN *et al.* 2002).

Following fertilization and embryonic development, *C. elegans* nematodes undergo four larval stages (L1, L2, L3, and L4) before maturing as adults (Figure 2A). The germlines of L4 larvae and adults contain cells at various stages of mitosis and meiosis. The HR machinery may be upregulated in meiotic cells that must repair numerous SPO-11-induced DSBs (ALPI *et al.* 2003), perhaps partially explaining the lack of sensitivity of L4 or adult germlines of NHEJ mutants to IR (Figure 3). To determine if HR is the main DNA DSB repair pathway used in the mitotic germline, L1 larvae that contain a small number of mitotically proliferating germ cells were examined (Figure 2A). Given that HR is essential in *C. elegans* (CHIN and VILLENEUVE 2001; ALPI *et al.* 2003), we chose to partially knock down HR gene products by RNAi. Although RNAi by feeding of HR genes resulted in inconsistent radiation hypersensitivity phenotypes for germlines of L1 larvae, consistent radiation-induced sterility was observed for RNAi of HR genes in an *rnf-3* genetic background, which is hypersensitive to RNAi (SIMMER *et al.* 2002). Further, the progeny of *rnf-3* animals fed *Escherichia coli* strains expressing double-strand RNA for several different HR genes typically displayed low levels of embryonic lethality and XO males (data not shown), phenotypes indicative of aneuploidy that might be expected if HR gene products were partially depleted and meiotic crossover formation was reduced. RNAi of the HR genes *rad-50*, *rad-51* (RINALDO *et al.* 2002; ALPI *et al.* 2003), *rad-54*, and *mre-11* (CHIN and VILLENEUVE 2001) resulted in severe hypersensitivity of germlines of L1 larvae to gamma irradiation at doses between 50 and 90 Gy, whereas animals with *lig-4* or *cku-80* mutations displayed wild-type levels of radiation sensitivity (Table 1). Moreover, mutations in *lig-4* or *cku-80* failed to enhance the radiation hypersensitivity phenotypes that occurred as a consequence of HR RNAi (Table 1), suggesting that even when HR function is compromised in mitotic germ cells, NHEJ plays little or no role in the repair of IR-induced DNA damage.

The above results suggest that HR may be the primary means of DSB repair in proliferating mitotic and meiotic germ cells in L4 larvae and adults. Note, however, that a role for NHEJ in DSB repair may have been missed if it were suppressed by modest levels of HR proteins present in HR RNAi strains whose germlines were highly sensitive to IR.

Noncycling germ cells in the late embryo use HR but not NHEJ to repair DSBs: After a *C. elegans* embryo is laid, a 3-hr period of rapid cell division occurs during

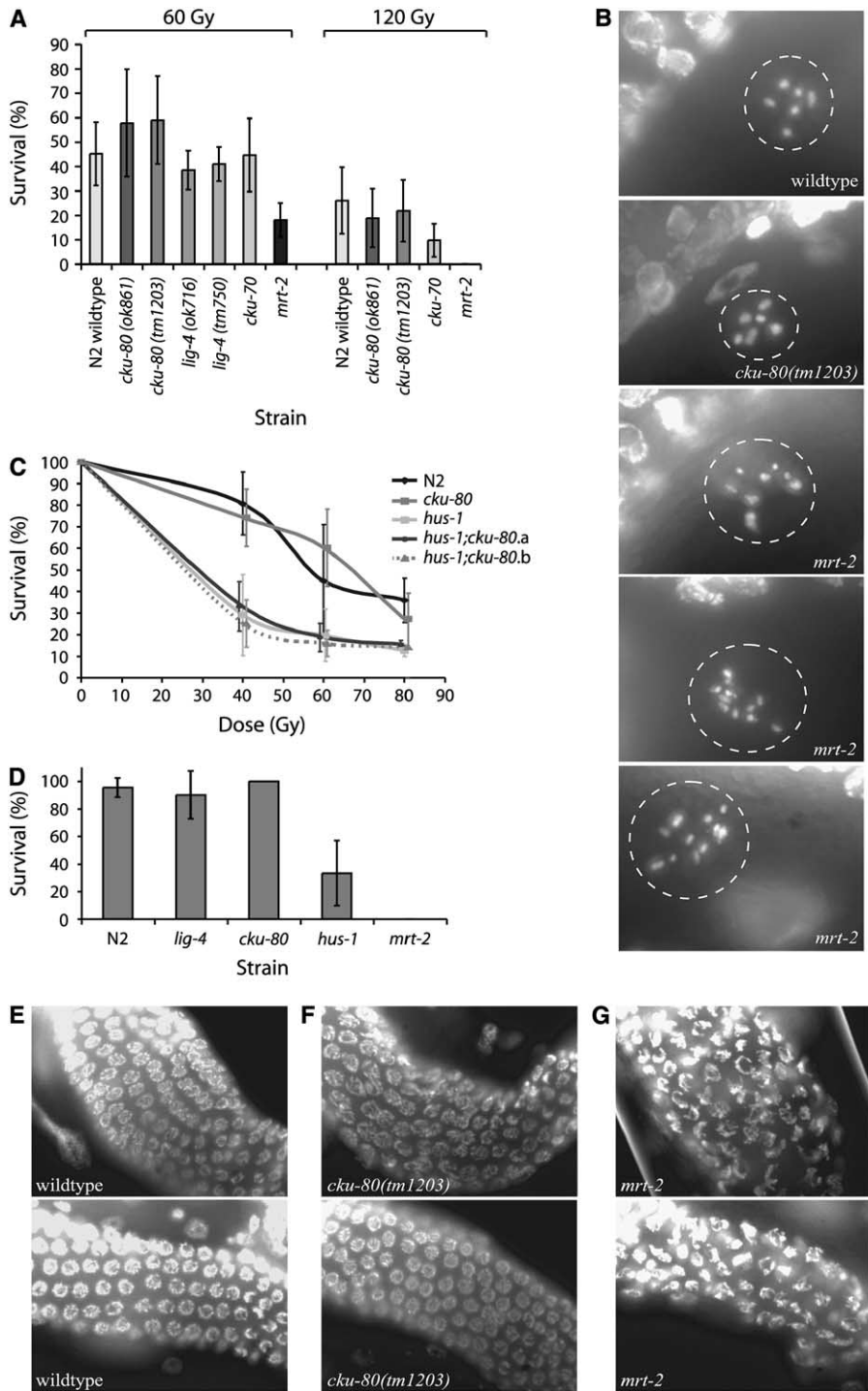


FIGURE 3.—Radiation response of NHEJ mutant germlines is normal. (A) NHEJ mutations do not significantly affect the survival of progeny of gamma-irradiated L4 larvae. (B) DAPI-stained oocyte nuclei (indicated by dashed circles) of adults derived from irradiated L4 larvae reveal a normally condensed complement of six bivalents for N2 wild-type and *cku-80* mutants but broken chromosomes for *mrt-2* strains. (C) NHEJ mutations such as *cku-80(ok861)* do not confer a role in germline DSB repair in the absence of the *hus-1* DNA damage response gene. Progeny of 10 worms from each strain were scored for viability as described (AHMED *et al.* 2001), which is a sensitive measure of genome instability in the *C. elegans* germline. (D) Arrested NHEJ mutant germ cells are not deficient for DSB repair. Progeny of adults derived from late-stage embryos irradiated with 50 Gy were scored for embryonic lethality. Error bars correspond to standard deviations. DAPI-stained germline nuclei of adults derived from late-stage embryos of (E) N2 wild type, (F) *cku-80*, or (G) *mrt-2* strains irradiated with 70 Gy. Pachytene nuclei at the border of the transition zone are shown.

early embryonic development (“early stage embryos”), followed by an 8-hr period that is virtually devoid of cell division (“late-stage embryos”) (Figure 2). Cell division resumes a few hours after the embryo hatches to become an L1 larva. Late-stage embryos deficient for HR genes displayed severe radiation-induced germline proliferation defects at or above 50 Gy and were uniformly sterile when they developed into adults (Table 2; data not shown). At an IR dose of 90 Gy, late-stage embryos harboring mutations in the *C. elegans* DNA damage

response genes *hus-1* and *mrt-2* developed into fertile adults whose progeny displayed high levels of embryonic lethality, which is a significant but weaker phenotype than the complete sterility observed when HR was knocked down. To assess if this apparently weaker phenotype might occur as a consequence of a lack of DNA-damage-induced apoptosis in the germlines of *hus-1* and *mrt-2* mutants (GARTNER *et al.* 2000; HOFMANN *et al.* 2002), *hus-1* or *mrt-2* strains were subjected to RNAi of *rad-51*, and, in this context, late-stage embryos

TABLE 1

Germline radiation sensitivity is displayed by L1 larvae deficient for HR or the DNA damage response, but not for L1 larvae deficient for NHEJ

Strain	0 Gy	30 Gy	50 Gy	90 Gy
N2	+++ ^a	+++	+++	++
<i>cku-80(tm1203)</i> on <i>vector</i> (RNAi)	+++	+++	+++	+
<i>lig-4(ok716)</i> on <i>vector</i> (RNAi)	+++	+++	+++	+
<i>mre-11</i> (RNAi)	+++	++	+	–
<i>lig-4(ok716)</i> on <i>mre-11</i> (RNAi)	+++	++	+	–
<i>rad-50</i> (RNAi)	+++	++	+	–
<i>lig-4(ok716)</i> on <i>rad-50</i> (RNAi)	+++	++	+	–
<i>rad-54</i> (RNAi)	+++	++	+	–
<i>lig-4(ok716)</i> on <i>rad-54</i> (RNAi)	+++	++	+	–
<i>rad-51</i> (RNAi)	+++	+	–	–
<i>lig-4(ok716)</i> on <i>rad-51</i> (RNAi)	+++	+	–	–
<i>cku-80(tm1203)</i> on <i>rad-51</i> (RNAi)	+++	+	–	–
<i>mrt-2</i>	+++	+	–	–

^a Germline DNA repair was scored qualitatively with respect to the number of surviving progeny derived from germlines of adults that develop from irradiated L1 larvae. Levels of survival are scored as +++ (wild type), ++ (weakly reduced), + (strongly reduced), or – (sterility or no survival).

irradiated with 50 Gy developed into completely sterile adults (data not shown). Thus, DNA-damage-induced apoptosis was not responsible for the more severe IR-induced phenotypes of strains deficient for HR. We conclude that the MRT-2 and HUS-1 proteins orchestrate checkpoint responses that enable optimal HR-mediated DSB repair to occur in developmentally arrested embryonic germ cells of late-stage embryos, but that significant levels of HR-mediated DSB repair occur in the absence of such DNA damage response proteins.

At doses >50 Gy, late-stage embryos with NHEJ defects incurred DNA damage that indirectly affected fertility via development of the somatic gonad (Table 2). Further, highly penetrant egg-laying defects of most adults derived from irradiated late-stage NHEJ mutant embryos precluded scoring progeny derived from their germ cells for survival (see below). For these reasons, a reduction in fertility at IR doses >50 Gy is difficult to interpret for adults derived from irradiated late-stage NHEJ mutant embryos. To assess if NHEJ might play a role in germ-cell DSB repair at high doses of irradiation, late-stage NHEJ mutant embryos were irradiated at 90 Gy and progeny of rare animals that developed into adults that laid eggs were scored for survival. In this case, progeny of irradiated late-stage NHEJ mutant and wild-type embryos displayed little or no embryonic lethality, whereas progeny of *hus-1* or *mrt-2* mutants displayed high levels of embryonic lethality, indicating DNA damage response defects (Figure 3D). Note that the rare NHEJ mutant embryos that developed into adults that laid eggs displayed strong IR-induced Gro and Unc phenotypes and often had somatic gonad damage, indicating that they had not escaped the effects of IR-induced DNA damage. Comparison of nuclear morphology of adults derived from irradiated late-stage NHEJ mutant embryos with adults derived from irradiated late-stage *rad-51*(RNAi) or *mrt-2* mutant embryos revealed severe aneuploidy and abnormal pachytene nuclei in *rad-51*(RNAi) and *mrt-2* germlines, whereas nuclei of NHEJ mutant and N2 wild-type adults displayed a normal, unirradiated nuclear morphology (Figure 3, E–G; Table 2; data not shown). Thus, neither microscopy-based nor progeny survival-based assays revealed a role for NHEJ proteins in DSB repair in germ cells of late-stage embryos at high doses of irradiation.

TABLE 2

Irradiated late-stage embryos of NHEJ mutants but not HR-depleted or DNA damage checkpoint mutation strains defective for *rad-51* or *mrt-2*, respectively, display severe defects in development of the somatic gonad but normal responses to DNA damage in their germlines

Dose	Strain	Mitotic failure	Aneuploidy in oocytes ^a	Egg-laying defect ^b	Severity of gonad defects ^c
0 Gy	N2 wild type	0/30	0/30	0/30	– (<i>n</i> = 30)
	<i>cku-80</i>	0/25	0/25	0/30	– (<i>n</i> = 25)
	<i>rad-51</i>	0/15	9/15	0/15	– (<i>n</i> = 15)
	<i>mrt-2</i>	0/20	0/20	0/25	– (<i>n</i> = 20)
90 Gy	N2 wild type	0/40	0/40	0/30	– (<i>n</i> = 40)
	<i>cku-80</i>	0/40	0/31	30/31	+++ (<i>n</i> = 40)
	<i>rad-51</i>	10/10	10/10	0/10	+ (<i>n</i> = 10)
	<i>mrt-2</i>	10/10	10/10	0/10	+ (<i>n</i> = 10)

^a Germline nuclear morphology (mitotic defects and aneuploidy) was judged by fluorescence microscopy.

^b Egg-laying defects are shown as a control for the level of somatic developmental defects in the mutant backgrounds.

^c The severity of gonad defects was based on how far the two distal tips of each gonad arm were from each other and on other positional defects, such as the failure of an arm to turn (+, modestly abnormal; +++, highly abnormal).

When late-stage *rad-51*(RNAi) embryos were irradiated with moderate doses of 10 or 20 Gy, progeny of adults derived from these embryos displayed intermediate levels of embryonic lethality, and an additional defect in *lig-4* failed to significantly enhance this

phenotype (data not shown). Thus, NHEJ does not function to repair IR-induced DNA damage in arrested embryonic germ cells of late-stage embryos, even when HR is compromised.

NHEJ is the main form of DSB repair in nondividing somatic cells: HR can provide error-free DSB repair, perhaps helping to ensure the integrity of the germ-cell lineage for future generations. In contrast, somatic cells are required for only a single generation and may be more tolerant of error-prone forms of DNA repair such as NHEJ. NHEJ mutants were therefore examined for defects in the repair of IR-induced DNA damage in the soma. For early stage embryos whose somatic cells proliferate rapidly (Figure 2), low doses of irradiation resulted in intermediate levels of lethality for wild-type or NHEJ mutant embryos, whereas depletion of the HR genes *rad-50*, *rad-51*, *rad-54*, and *mre-11* resulted in increased levels of embryonic lethality (Figure 4A; data not shown). The absence of NHEJ in HR-depleted backgrounds failed to enhance their hypersensitivity to IR (Figure 4A), suggesting a nonredundant role for HR repair of IR-induced DNA damage in the early embryo.

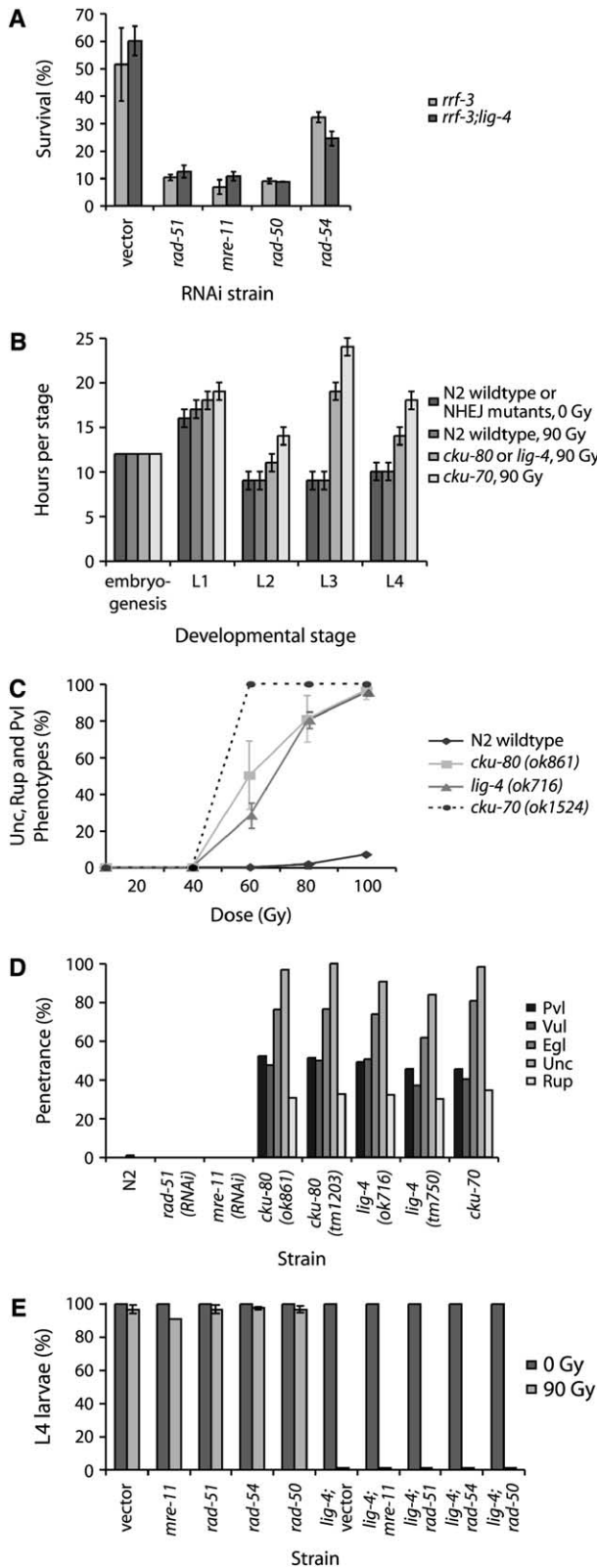


FIGURE 4.—Somatic functions for HR and NHEJ in early or late embryogenesis, respectively. (A) *rrf-3* control or *rrf-3; lig-4(ok716)* *C. elegans* strains were fed on bacteria expressing double-stranded RNA for vector or for *rad-51*, *mre-11*, *rad-50*, or *rad-54* HR genes, and early stage F₂ embryos derived from F₁ progeny were irradiated at 30 Gy. Survival was computed relative to unirradiated controls as the ratio of live worms to total worms in irradiated samples divided by the ratio of live worms to total worms in the unirradiated samples. Averages for two experiments are shown. Late Egg Rad phenotypes of NHEJ mutants are shown as (B) the Gro phenotype; (C) combined totals for Unc, Rup, and Pvl phenotypes; and (D) individual totals for Pvl, Vul, Egl, Rup, and Unc phenotypes. For B, developmental stages were scored by monitoring embryos and larvae every 2 hr, from the time of irradiation until adulthood. Larval stages were defined as initiating when >90% of the worms had reached a stage. Results in C are shown for representative experiments that were repeated at least twice for *cku-80(ok861)* and *lig-4(ok716)* with similar results. (D) N2 wild type, *cku-80(ok861)*, *lig-4(ok716)*, *cku-80(tm1203)*, *lig-4(tm750)*, and *cku-70(ok1524)* were irradiated at 90 Gy and scored as described in MATERIALS AND METHODS (*n* = 91, 96, 101, 116, and 87, respectively). Note that the Rup phenotype was larval lethal and that the percentages of Pvl, Vul, and Egl phenotypes were derived exclusively from animals that survived until adulthood. *rad-51*(RNAi) or *mre-11*(RNAi) strains did not display Late Egg Rad phenotypes. (E) Deficiency for HR genes does not cause a Slow Growth phenotype in irradiated late-stage embryos, nor does it suppress the Slow Growth phenotype of NHEJ mutants such as *lig-4(ok716)*. Experiments were conducted in *rrf-3* or *rrf-3; lig-4* genetic backgrounds, and the percentage of larvae that had reached the L4 stage was scored at 48 hr post-irradiation. The effectiveness of the HR gene RNAi was confirmed by observing complete sterility for irradiated late-stage embryos. *n* = 100 scored for each strain, except for *mre-11*(RNAi) (*n* = 61 at 0 Gy, *n* = 64 at 90 Gy), *rad-54*(RNAi);*lig-4* (*n* = 95 at 0 Gy, *n* = 84 at 90), and *rad-50*(RNAi);*lig-4* (*n* = 62 at 90 Gy). Error bars correspond to standard deviations.

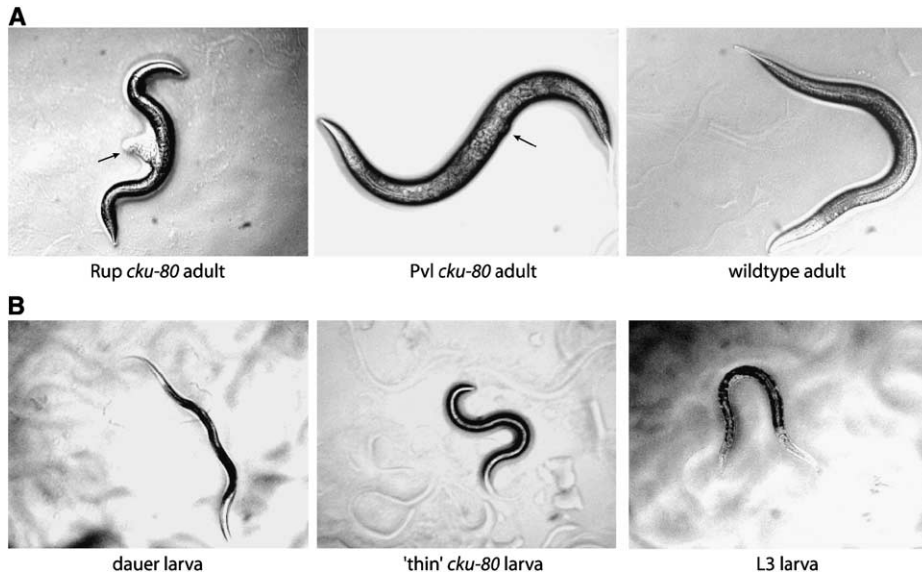


FIGURE 5.—Phenotypes of late-stage embryos irradiated with 70 Gy IR. (A) Adult phenotypes. Arrows indicate gonad protruding from ruptured vulva (Rup) or protruding vulva (Pvl). (B) Irradiated late-stage embryos of NHEJ mutants can give rise to thin larvae in comparison to an irradiated N2 wild-type control. An unirradiated *daf-2(m41)* dauer larva is shown for comparison.

While irradiation of early stage embryos (Early Egg Rad assay) (Figure 2) with doses >50 Gy resulted in almost complete embryonic lethality for wild type, no embryonic lethality was observed when late-stage embryos composed of noncycling cells were irradiated (Late Egg Rad assay) with IR doses of up to 140 Gy, even when NHEJ or HR were deficient (data not shown). The embryonic lethality observed for irradiated early stage embryos (Figure 4A) may therefore occur as a consequence of cell proliferation.

Gamma irradiation of late-stage NHEJ mutant embryos did result in an increase in the frequency and magnitude of several postembryonic developmental phenotypes (Figure 4, B–D). The most pronounced Late Egg Rad phenotypes were Gro (Figure 4B), Protruding Vulva (Pvl), Vul, Ruptured (Rup), Unc, and Egg-Laying Defective (Egl) (Figure 4, C and D; Figure 5A). When NHEJ mutants were irradiated in late embryogenesis, the time to hatching was unaffected (Figure 4B), suggesting that cell proliferation may be required for the Gro phenotype to occur. Minor growth delays were observed for L1 and L2 larval stages. The greatest delays in growth rate were observed during the L3 and L4 larval stages (30 and 40 hr post-irradiation) (Figure 4B), during which time the last somatic postembryonic cell divisions occur (SULSTON and HORVITZ 1977). Analogously, defects in mammalian DNA repair genes, including *DNA ligase IV* mouse mutants, result in developmental delay and neuronal defects (O'DRISCOLL *et al.* 2004). Such abnormalities occur in the absence of exogenous DNA damage, precluding a direct comparison with the phenotypes of irradiated *C. elegans* NHEJ mutants. However, a similar molecular trigger may be responsible for the resulting developmental defects.

Mice heterozygous for mutations in *DNA ligase IV* or *Ku86* have been shown to display chromosome instability (KARANJAWALA *et al.* 1999). Further, haplo-

insufficiency for *DNA ligase IV* in an *Ink4a* mutant background increases the risk of tumor development in mice (SHARPLESS *et al.* 2001), and heterozygosity for NHEJ components has been suggested as a predictor of human disease. Late-stage embryos of *C. elegans* strains that were heterozygous or multiply heterozygous for *cku-80* or *lig-4* failed to display somatic NHEJ defects (Table 3; data not shown), indicating that NHEJ in *C. elegans* is not haplo-insufficient. Deletion alleles of either *cku-80* or *lig-4* failed to complement independent deletion mutations in *cku-80* or *lig-4*, respectively, for the IR-induced phenotypes, which are therefore likely to occur as a consequence of defects in these canonical NHEJ genes (Table 3). *cku-70* had a higher penetrance of Late Egg Rad phenotypes than alleles of *cku-80* or *lig-4* (Figure 4, C and D). These effects could be due to a secondary mutation in a tightly linked gene that synergizes with NHEJ defects (see below) and may have

TABLE 3

Larvae derived from late-stage NHEJ mutant embryos exhibit a radiation-induced Gro phenotype

Strain	% at L4 stage (90 Gy)	% at L4 stage (0 Gy)
N2	100 ($n = 81$)	100
<i>cku-80(ok861)</i>	0 ($n = 72$)	100
<i>cku-80(tm1203)</i>	0 ($n = 65$)	100
<i>lig-4(ok716)</i>	0 ($n = 67$)	100
<i>lig-4(tm750)</i>	0 ($n = 61$)	100
<i>cku-70(tm1524)</i>	0 ($n = 70$)	100
<i>cku-80(ok861)/cku-80(tm1203)</i>	0 ($n = 70$)	100
<i>lig-4(ok716)/lig-4(tm750)</i>	0 ($n = 29$)	100
<i>cku-80(ok861)/+</i>	100 ($n = 61$)	100
<i>lig-4(ok716)/+</i>	100 ($n = 32$)	100
<i>cku-80(ok861)/lig-4(ok716)</i>	100 ($n = 54$)	100

been responsible for the modest increase in the sensitivity of germlines of *cku-70* L4 larvae to IR at 120 Gy (Figure 3A). Alternatively, *cku-70* may have an additional repair function, a possibility that awaits recovery of a second allele of *cku-70* for clarification. In this context, a study in *S. cerevisiae* reported that *hdf1/yku70* mutants are more sensitive than *hdf2/yku80* mutants to the radiomimetic chemicals bleomycin and methyl methanesulfonate (FELDMANN *et al.* 1996), and KU70 in mammalian cells has functions that are distinct from KU80 (COHEN *et al.* 2004). To confirm the above results, RNA interference was performed for *cku-70*, *cku-80*, and *lig-4* by both feeding and injection, but consistent radiation-induced phenotypes were not observed (data not shown). Note that RNAi of many DNA damage response genes results in incompletely penetrant phenotypes (BOULTON *et al.* 2002).

Deficiency for *DNA ligase IV* in a chicken cell line resulted in a high level of IR-induced lethality that was partially rescued by mutation of *KU70*, possibly because the DSB-binding Ku heterodimer may prevent the HR machinery from processing unrepaired DSBs in the absence of *DNA ligase IV* (ADACHI *et al.* 2001). Further, deficiency for *DNA ligase IV* in mice results in embryonic lethality, which is rescued by mutation of *Ku86* (KARANJAWALA *et al.* 2002). However, the Gro phenotype of late-stage *lig-4;cku-80* double-mutant embryos irradiated with 50 Gy was not significantly different from that of either single mutant (*lig-4;cku-80* = 44 ± 5% L4; *cku-80* = 36 ± 18% L4; *lig-4* = 32 ± 7% L4; wild type = 100% L4; *n* = 100 each), suggesting that the two genes may act in the same pathway of DSB repair. Thus, the absence of *lig-4* in *C. elegans* late-stage embryos does not result in unrepaired DSBs that are substrates for HR. Although IR-induced DNA damage results in a variety of lesions, including both single- and double-strand DNA breaks, the conclusion that unrepaired IR-induced DSBs are likely to trigger the developmental phenotypes of irradiated late-stage NHEJ mutant *C. elegans* embryos is based, in part, on synergy observed with a *him-10* kinetochore mutation (see below). In addition, a large body of evidence from other systems suggests that the primary function of canonical NHEJ proteins is to repair DNA double-strand breaks (LIEBER *et al.* 2003). Note that DSB repair assays are always biased by the biological, chemical, or physical means by which DNA breaks are induced, and ionizing radiation is currently the most effective method for evenly distributing DSBs in germ and somatic cells at multiple stages of *C. elegans* development.

In contrast to NHEJ mutants, worms deficient for the *rad-50*, *rad-51*, or *rad-54* HR genes exhibited no somatic Late Egg Rad phenotypes (Figure 4, D and E; Table 4; data not shown). This was also the case for *mre-11*, which is known to be important for both HR and canonical NHEJ in *S. cerevisiae*, but is thought to function primarily in HR in other organisms (DALEY *et al.* 2005). Further,

TABLE 4

HR mutants do not exhibit Late Egg Rad phenotypes

Strain	Pvl/total (90 Gy)	Unc/total (90 Gy)	Unc/total (0 Gy)
N2	0/55	0/55	ND
<i>rad-51;dpy-13^{a,b}</i>	0/20	0/20	ND
<i>cku-80;dpy-13^b</i>	24/51	51/51	ND
<i>mre-11/nT1 F1^{a,c}</i>	0/105	92/105	182/190

^a *mre-11* and *rad-51* progeny of heterozygous parents were irradiated as late-stage embryos.

^b Although *rad-51* was marked *in cis* with *dpy-13*, the *dpy-13* mutation did not suppress the Pvl phenotype in a *cku-80* background.

^c *mre-11* was maintained as a heterozygote using an *nT1* translocation that confers a dominant Unc phenotype, such that non-Unc progeny were *mre-11* homozygotes. Irradiation failed to increase the frequency of Unc animals.

deficiency for *rad-50*, *rad-51*, *rad-54*, or *mre-11* failed to enhance or suppress somatic Late Egg Rad phenotypes of either *cku-80* or *lig-4* (Figure 4E; data not shown), indicating that HR has no impact on phenotypes that result from radiation-induced DSBs in nondividing somatic cells, even in the absence of NHEJ. Although DNA damage checkpoint genes have been reported to confer modest alterations in NHEJ-mediated DSB repair in *S. cerevisiae* (DE LA TORRE-RUIZ and LOWNDES 2000), Late Egg Rad phenotypes were not observed when late-stage *mrt-2* or *hus-1* DNA damage checkpoint mutant embryos were irradiated (data not shown).

NHEJ-mediated DSB repair occurs in somatic but not germ cells of dauer larvae: Given that NHEJ is an important DNA repair process in nondividing somatic cells of late-stage *C. elegans* embryos (Figure 4, B–D), the impact of NHEJ on DSB repair was examined for dauer larvae, as neither somatic nor germline cells proliferate at this larval stage. Stress-resistant dauer larvae occur when conditions such as starvation, high population density, or high temperature are encountered, and dauers can remain developmentally arrested for months (Figure 2A) (RIDDLE *et al.* 1981). Given that NHEJ-mediated DNA repair is enhanced 50-fold in starved/stationary cells in *S. cerevisiae* (KARATHANASIS and WILSON 2002), *C. elegans* dauer larvae, which are induced by stresses such as starvation, might be expected to utilize NHEJ as their major DSB repair pathway. To obtain significant quantities of dauer larvae for our experiments, conditional dauer-constitutive alleles of the *daf-2* insulin-signaling and *daf-8* TGF- β pathway genes were used (MALONE and THOMAS 1994).

Although irradiated control *daf-8* and *daf-2* dauers displayed no lethality, ~50% of irradiated NHEJ-defective *daf-8* dauers and 10% of irradiated NHEJ-defective *daf-2* dauers died before exiting the dauer stage (Figure 6). NHEJ mutant dauers that survived irradiation and proceeded to develop into adults displayed Pvl, Vul, Rup,

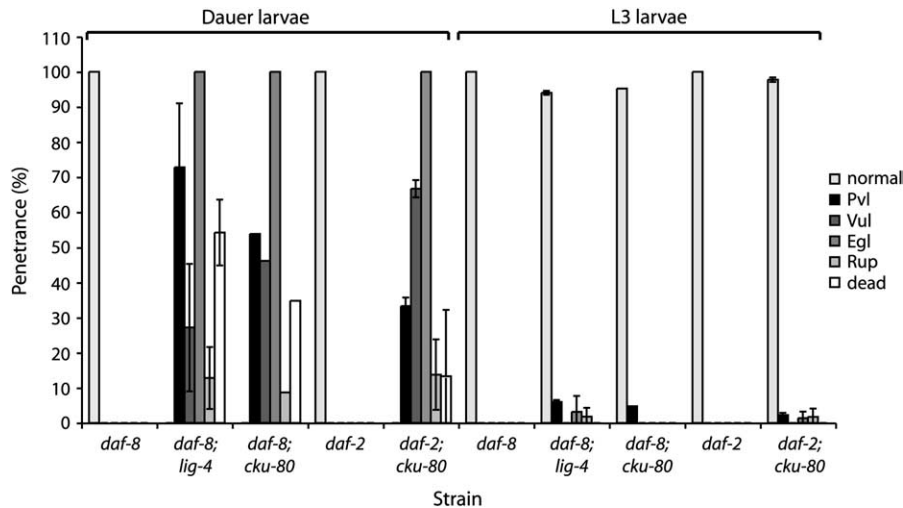


FIGURE 6.—Irradiated NHEJ mutant dauers exhibit some phenotypes of irradiated late-stage NHEJ mutant embryos. The mutations were *cku-80(ok861)*, *lig-4(ok716)*, *daf-8*, and *daf-2(m41)*. Similar results were obtained with *daf-2(e1368)*. Dauer larvae were *daf-8* ($n = 21$ and 25), *lig-4;daf-8* ($n = 46$ and 21), *cku-80;daf-8* ($n = 23$), *daf-2* ($n = 55$ and 30), and *daf-2;cku-80* ($n = 48$ and 30). L3 larvae were *daf-8* ($n = 30$), *daf-8;lig-4* ($n = 47$ and 56), *daf-8;cku-80* ($n = 42$), *daf-2* ($n = 30$ and 33), and *daf-2;cku-80* ($n = 59$). Results were averaged from two experiments and error bars correspond to standard deviations.

and Egl phenotypes (Figure 6) but not the Unc or Gro phenotypes observed for irradiated late-stage NHEJ mutant embryos (data not shown). Thus, the developmental defects that cause the Unc and Gro phenotypes may occur prior to the L3 larval stage, which is consistent with irradiated late-stage NHEJ mutant embryos displaying a weak Slow Growth phenotype at the L1 and L2 larval stages and the Unc phenotype at the L1 stage (Figure 4B; data not shown). Further, the lack of an Unc phenotype following irradiation of NHEJ mutant dauers agrees with the observation that many neurons are postmitotic after the L2 stage (prior to dauer entry) (SULSTON and HORVITZ 1977). In contrast, the vulval cell lineage remains mitotic until mid-L4 (after dauer exit) (SULSTON and HORVITZ 1977), which is consistent with the radiation-induced Pvl, Vul, and Egl developmental defects of NHEJ mutant dauer larvae. The Egl phenotype may occur as a consequence of the Pvl and Vul defects, which are probably two manifestations of a single defect in vulval induction, since the combined penetrance of these phenotypes at 90 Gy was $\sim 100\%$ (Figures 4D and 6).

The IR dose at sterility for *mrt-2* checkpoint-defective and wild-type dauers was 100 and 220 Gy, respectively. In contrast, NHEJ-defective dauers became sterile at a dose of 200 Gy, indicating that DSB repair of germ cells in dauer larvae is not significantly affected by NHEJ. The slightly lower dose at sterility of NHEJ-defective dauers probably reflects IR-induced defects in development of the somatic gonad (Table 2) rather than germline defects, because most or all embryos laid by adults derived from irradiated control or NHEJ mutant dauers hatched at doses immediately below sterility, whereas embryos of adults derived from irradiated *mrt-2* or *hus-1* checkpoint-defective dauers displayed high levels of DNA-damage-induced lethality (data not shown). Thus, the NHEJ pathway is used by vulval somatic cells but not by germ cells for repair of IR-induced DNA damage in dauer larvae.

Noncycling germ cells arrest in late S- and G₂-phases:

Studies in yeast and mammalian cells indicate that HR is enhanced in late S- and G₂-phases of the cell cycle (TAKATA *et al.* 1998; KARATHANASIS and WILSON 2002; FERREIRA and COOPER 2004) and that upregulation of HR occurs as a consequence of cyclin-dependent kinase activity (AYLON *et al.* 2004; FERREIRA and COOPER 2004). Thus, a difference in the phase of cell cycle arrest might provide a plausible explanation for why developmentally arrested germ but not somatic cells depend on HR for DSB repair. Quantification of the DNA-binding dye DAPI in nuclei of arrested germ cells of dauer larvae revealed that they had a mean of 2.03 ± 0.34 times the DNA content of somatic nuclei found in the ventral nerve cord and vulva ($n = 20$ each), indicating that germ cells arrest at G₂ with a 4N DNA content, whereas somatic cells arrest at G₁ with a 2N DNA content. In contrast, polyploid intestinal nuclei of dauers had a mean of 4.05 ± 0.35 times the DNA content of other somatic nuclei ($n = 20$ each), indicating that they arrest with genome copy number of 8N.

We attempted to measure the DNA content of nuclei of the two germ cells, Z2 and Z3, in late-stage *C. elegans* embryos. These nuclei were identified using the antibody OIC1D4 that binds to P-granules, which specify *C. elegans* germ cells (STROME and WOOD 1982). Although nuclei of late-stage embryos were too crowded to obtain accurate measurements of DNA content, an antibody to phospho-histone H3, a marker of mitosis, failed to label arrested germ cells in late-stage embryos, whereas it did label nuclei in early stage embryos experiencing high levels of cell proliferation (data not shown). Further, chromosomes of germ-cell nuclei of late-stage embryos were not condensed. Together, these observations suggest that embryonic germ cells arrest in interphase.

Cells of late-stage *C. elegans* embryos remain quiescent until several hours after well-fed L1 larvae hatch (Figure 2B). For L1 larvae whose development is arrested immediately after hatching as a consequence of

TABLE 5

Genes that participate in various biological pathways that failed to suppress somatic phenotypes of irradiated late-stage NHEJ mutant embryos

Gene tested	Biological process	Mutant ^a	RNAi ^a
<i>ced-3</i>	Apoptosis	+	-
<i>cep-1</i> (p53)	DNA damage checkpoint	+	+
<i>phg-1</i>	DNA damage checkpoint	-	+
<i>hus-1</i>	DNA damage checkpoint	+	-
<i>rad-5/clk-2</i>	DNA damage and S-phase checkpoints	+	-
<i>atm-1</i>	DNA damage sensor	-	+
<i>atl-1</i>	DNA damage sensor	-	+
<i>bub-1</i>	Mitotic checkpoint	-	+
<i>lin-35</i> (Rb)	G ₁ /G ₂ checkpoint	-	+
<i>daf-12</i>	Stress response	+	-
<i>daf-16</i>	Stress response	+	-

^a Genetic mutations (+) or RNAi-mediated depletion (+) were used to test for suppression in *cku-80(tm1203)* or *lig-4(tm716)* backgrounds. - (not tested).

starvation, most somatic nuclei have a 2N DNA content (HEDGECOCK and WHITE 1985). Thus, it is likely that somatic cells of late-stage *C. elegans* embryos arrest in G₁. In contrast, germ cells of starved L1 larvae have been shown to arrest in G₂ with a 4N DNA content (M. FUKUYAMA and J. ROTHMAN, personal communication). We conclude that germ cells in both late-stage embryos and dauer larvae are likely to undergo developmentally induced cell cycle arrest in G₂-phase of the cell cycle, in contrast to their somatic neighbors, which arrest in G₁.

Suppression of somatic Late Egg Rad phenotypes:

Mice deficient for *DNA ligase IV* die as embryos as a result of neuronal apoptosis that may occur as a consequence of improper repair of endogenous DSBs (BARNES *et al.* 1998). It is possible that *C. elegans* somatic cells with unrepaired DSBs might undergo apoptosis, which could trigger one or more of the Late Egg Rad phenotypes observed for NHEJ mutants. Null mutations in either the *ced-3* caspase, which is essential for apoptosis (YUAN *et al.* 1993), or the *C. elegans* p53 ortholog *cep-1*, which is required for DSB-induced apoptosis in the germline (DERRY *et al.* 2001; SCHUMACHER *et al.* 2001), failed to suppress any Late Egg Rad phenotype observed in NHEJ mutants (Table 5). We conclude that DNA-damage-induced apoptosis does not cause the Late Egg Rad phenotypes of NHEJ mutants, consistent with the previous observation that ionizing radiation does not induce apoptosis in *C. elegans* somatic cells (GARTNER *et al.* 2000).

The radiation-induced developmental defects of NHEJ mutants might have been caused by DNA-damage-mediated activation of the S-phase checkpoint or the G₂/M DNA damage checkpoint, which can slow or halt progression of the cell cycle (SANCAR *et al.* 2004). However, mutations that affect these processes, such as

rad-5/clk-2 or *hus-1* (GARTNER *et al.* 2000; AHMED *et al.* 2001; HOFMANN *et al.* 2002), failed to suppress the Late Egg Rad phenotypes of irradiated late-stage NHEJ mutant embryos (Table 5). It is also possible that the myriad Late Egg Rad phenotypes observed for NHEJ mutants might have been caused by activation of the mitotic checkpoint monitoring segregation of unrepaired chromosome fragments. However, RNAi-mediated depletion of the mitotic checkpoint gene *bub-1* induced the expected developmental defects of embryonic lethality, protruding vulva, and ruptured vulva in the absence of irradiation (FRASER *et al.* 2000; SIMMER *et al.* 2003), but failed to suppress any radiation-induced Late Egg Rad phenotype (Table 5).

IR treatment of late-stage NHEJ mutant embryos could activate stress response pathways. For NHEJ mutant strains irradiated as late-stage embryos, 2–5% of developing larvae were thin at the L3 stage and resembled dauer larvae, an alternative larval stage that can be induced by various stresses (Figure 5B). However, the thin larvae failed to undergo developmental arrest when singled, as might be expected for dauer larvae, suggesting that they may transiently adopt dauer characteristics. However, unlike partial dauers (VOWELS and THOMAS 1992), these thin animals did not have alae or a constricted pharynx (data not shown). The Dauer phenotype is known to be intrinsically temperature sensitive for both wild-type and dauer-constitutive mutant strains, which both show enhanced induction of dauer formation when grown at increasing temperatures. However, the frequency of thin larvae from irradiated NHEJ mutant embryos was the same at both 20° and 25° (data not shown). Moreover, neither the thin dauer-like phenotype nor any other somatic phenotype observed upon irradiation of late-stage NHEJ mutant embryos was suppressed by mutations in either *daf-12* or *daf-16* (Table 5), which are essential for dauer formation (VOWELS and THOMAS 1992).

It was previously reported that L1 larvae of *C. elegans* strains deficient for the Werner's helicase *wrn-1* display a radiation-induced Egl phenotype that can be suppressed by the neurotransmitter serotonin (LEE *et al.* 2004). Given that WRN has been shown to physically interact with Ku (COOPER *et al.* 2000), the Egl phenotype that occurs in irradiated late-stage NHEJ mutant embryos might be similar to that previously observed for *wrn-1* mutants. However, *wrn-1* mutants failed to display Late Egg Rad phenotypes, although irradiation of L1 larvae produced weak effects, as previously reported (LEE *et al.* 2004). The possibility that the radiation-induced Egl defect of NHEJ mutants was caused by a defect in neurons innervating egg-laying muscles was examined using an excess of serotonin, but this failed to rescue the Egl phenotype (Figure 7) (BASTIANI *et al.* 2003). Taken together, these results indicate that *wrn-1* is not involved in NHEJ in *C. elegans* and that the physiological basis of the radiation-induced Egl phenotype

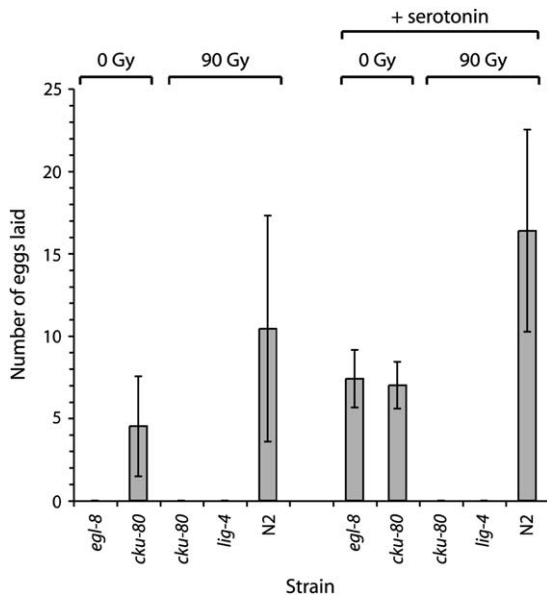


FIGURE 7.—Serotonin does not rescue the egg-laying defect of irradiated NHEJ mutants. An *egl-8* mutant strain, which has an Egl phenotype as a consequence of a neurotransmission defect and is rescued by serotonin (BASTIANI *et al.* 2003), is shown as a control. Ten gravid adults from each sample were placed in individual wells containing 7.5 mM serotonin (5-hydroxytryptamine; Sigma, St. Louis) for 90 min and eggs were counted as described (BASTIANI *et al.* 2003).

of NHEJ mutants is different from that reported for the *wrn-1* Egl phenotype (LEE *et al.* 2004). Defects in vulval morphogenesis may be responsible for the radiation-induced NHEJ mutant Egl phenotype (Figure 4D).

In summary, the observed Late Egg Rad phenotypes of NHEJ mutants were not caused by apoptosis, the stress-induced dauer pathway, the S-phase checkpoint, the DNA damage checkpoint, or the spindle checkpoint.

Intestinal cells exhibit karyokinesis defects: *C. elegans* intestinal nuclei undergo several rounds of endoreduplication during development to reach a final copy number of 32N, and they are therefore easily distinguished from other nuclei on the basis of their DNA content. A high frequency of intestinal nuclei that had failed to divide was observed in DAPI-stained L2 larvae derived from irradiated late-stage NHEJ mutant embryos (Figure 8, A and B). The identity of these nuclei was confirmed using an *elt-2::GFP* transgene that is expressed only in intestinal nuclei (FUKUSHIGE *et al.* 1999). GFP-positive intestinal nuclei of irradiated *elt-2::GFP;lig-4* or *elt-2::GFP;cku-80* doubles failed to complete nuclear division by the end of the L1/L2 larval molt, whereas irradiated *elt-2::GFP* controls had completed nuclear division by this time (Figure 8, C–E). Nuclei of the six anterior intestinal cells, which do not undergo postembryonic nuclear divisions, were always observed as single nuclei in irradiated NHEJ mutants. Further, aberrant nuclei were never observed prior to nuclear division of the posterior intestinal cells (data

not shown). Thus, IR treatment of late-stage NHEJ mutant embryos interfered with the postembryonic nuclear divisions of posterior intestinal cells, which occur immediately prior to the L1/L2 molt, and persisted in L4 larvae and adults (data not shown). The failure of intestinal nuclei to divide suggests a defect in chromosome segregation that may reflect an additional form of IR-induced somatic damage that initiates in the first larval stage.

Kinetochores defects synergize with somatic phenotypes of irradiated NHEJ mutants: Analysis of many DAPI-stained larvae and adults derived from irradiated late-stage NHEJ embryos failed to reveal mis-segregating chromosome fragments, with the exception of the chromatin bridges observed between dividing polyploid intestinal nuclei in adults (Figure 8 and data not shown). Despite failing to observe additional chromosome mis-segregation events directly, RNAi-mediated depletion of 29 *C. elegans* genes involved in chromosome segregation revealed that 19 exhibited Pvl, Vul, Gro, Rup, and/or Unc phenotypes, whereas the rest either had no RNAi phenotype or were Embryonic Lethal (MADDOX *et al.* 2004). These phenotypes were reminiscent of the Late Egg Rad phenotypes of NHEJ mutants. In a mutant background defective for kinetochore attachment to microtubules, one might expect mis-segregation of chromosome fragments to be exacerbated in irradiated NHEJ mutant embryos, as poleward mitotic chromosome segregation forces are probably proportional to the number of microtubules that can attach to a holocentric kinetochore of a *C. elegans* chromosome, and this number is proportional to the length of a chromosome. To test this hypothesis, double mutants containing defects in both NHEJ and the *him-10* kinetochore protein (HOWE *et al.* 2001) were constructed. At 30 Gy, no Late Egg Rad phenotypes were observed for either NHEJ or *him-10* single mutants, whereas significant frequencies of Gro, Pvl, Vul, and Unc phenotypes were observed when late-stage *him-10;cku-80* double-mutant embryos were irradiated (Figure 9). Similar results were obtained for *him-10;cku-70* double mutants (data not shown). Thus, mutation of the *him-10* kinetochore component was synergistic with NHEJ defects for hypersensitivity of noncycling somatic cells to ionizing radiation, which is therefore likely to result from chromosome mis-segregation events that occur as a consequence of unrepaired DNA DSBs. Further, our results corroborate suspicions that the cause of the panoply of developmental defects that occur in *C. elegans* strains deficient for kinetochore or mitotic proteins is chromosome mis-segregation.

DISCUSSION

We have shown that NHEJ is the major pathway for repair of radiation-induced DNA damage in quiescent somatic cells in *C. elegans* embryos and dauer larvae, whereas HR is used for DNA repair in proliferating

somatic cells and in germ cells at all stages of development. Given that NHEJ is used throughout the cell cycle in vertebrate cells, it was expected that NHEJ or HR might be able to substitute for one another if one of these pathways is compromised. However, our results suggest that in rapidly proliferating somatic cells of early-stage embryos, in nondividing germ or somatic cells of late-stage embryos, in germ cells of L1 larvae, and in germ cells of L4 larvae and adult *C. elegans* nematodes, HR and NHEJ may be nonredundant. A recent study

supports this possibility, as *lig-4*-mediated DSB repair in the adult *C. elegans* germline is apparent only in the absence of both HR and an additional DSB repair pathway (MARTIN *et al.* 2005). We have extended the observation that NHEJ is repressed in the adult germline to dividing and nondividing germ cells at earlier stages of development (Table 1). Thus, meiotic development is not a prerequisite for this property.

Consistent with our observations, *P*-element-induced DSBs are preferentially repaired by HR in mitotic germ cells of *Drosophila* adults, whereas most somatic *P*-element-induced DSBs are repaired by NHEJ (ENGELS *et al.* 1990; JOHNSON-SCHLITZ and ENGELS 1993; GLOOR *et al.* 2000). Further, HR is used for DNA repair during the initial series of rapid embryonic nuclear divisions following fertilization of a *Drosophila* embryo, similar to the results that were observed for early stage *C. elegans* embryos (Figure 4). HR, and to a lesser degree NHEJ, are used to repair IR-induced DNA damage at later stages of *Drosophila* development, although it is unclear how this division of labor is partitioned (GORSKI *et al.* 2003). Our results suggest that this overlap of DNA repair pathways may reflect a mixed population of replicating and quiescent cells that rely on HR and NHEJ, respectively. Some *P*-element-induced DSBs are repaired by NHEJ in the mitotic germline of *Drosophila* adults (JOHNSON-SCHLITZ and ENGELS 1993), but DNA ligase IV is not required to seal such breaks, which instead result from microhomology-mediated rather than canonical end joining (McVEY *et al.* 2004). Although Mre11 is required for microhomology-mediated end joining in yeast and plants (MA *et al.* 2003; HEACOCK *et al.* 2004), *mre-11* deficiency did not confer an additional somatic DSB repair defect for NHEJ-defective late-stage *C. elegans* embryos (Figure 4D), indicating that this noncanonical end-joining pathway is not a primary or alternative end-joining pathway in this context.

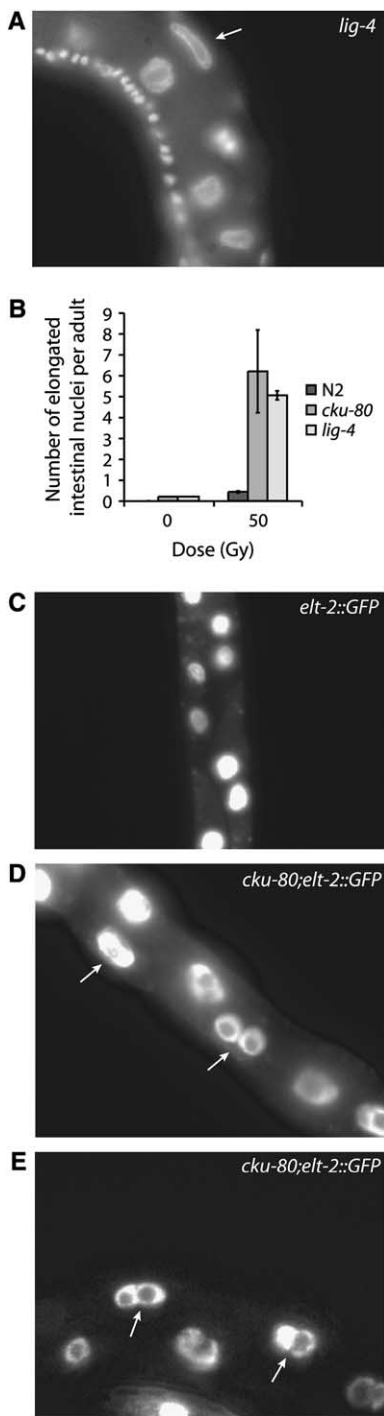


FIGURE 8.—Karyokinesis failure of intestinal nuclei in larvae derived from late-stage NHEJ mutant embryos. (A) Example of an elongated intestinal nucleus observed in a DAPI-stained L2 larva derived from an irradiated *lig-4(ok716)* mutant late-stage embryo. Several normal polyploid intestinal nuclei are observed as large, round halos, whereas an elongated intestinal nucleus is also observed (arrow). A string of diploid ventral nerve cord nuclei are observed on the left side of the larva. (B) Quantification of karyokinesis defects in N2 wild-type, *cku-80(tm1203)*, or *lig-4(ok716)* strains. The number of elongated nuclei per DAPI-stained adult derived from late-stage embryos is indicated ($n = 20$ /strain, with each experiment repeated twice). Mean scores are shown and error bars represent standard deviations. (C) An *elt-2::GFP* reporter labels intestinal nuclei, which normally complete nuclear division and clearly separate in L2 larvae derived from irradiated late-stage embryos, as shown. (D and E) In a *cku-80(tm1203);elt-2::GFP* strain, intestinal nuclei with karyokinesis defects (arrows) are observed after the L1/L2 molt in larvae derived from irradiated late-stage embryos.

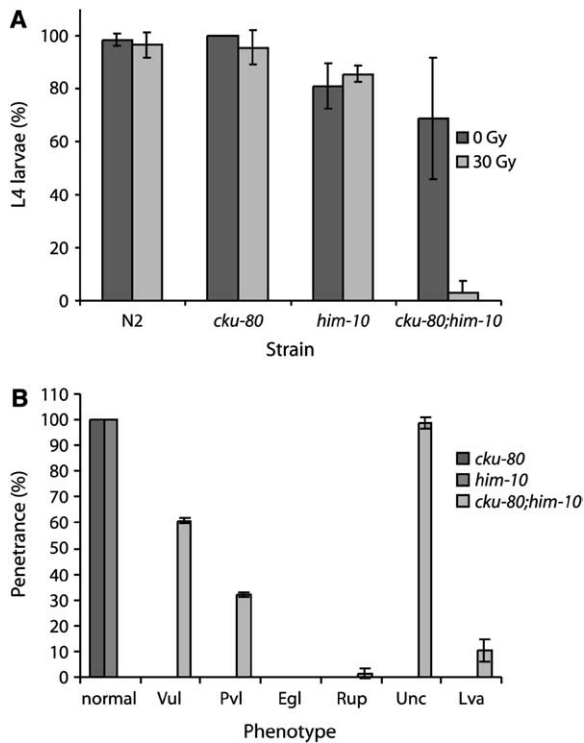


FIGURE 9.—Synergy of NHEJ mutations with *him-10* at 30 Gy. (A) The Gro phenotype was scored as the percentage of larvae that had reached the L4 stage 48 hr after their embryos were laid ($n = 64$ and 72 for *cku-80*; $n = 53$ and 70 for *him-10*; $n = 36$ and 70 for *cku-80;him-10*). (B) Other Late Egg Rad phenotypes were scored for the same strains ($n = 50$ /strain/experiment). Egl worms were not observed either in *him-10;cku-80* double mutants or in *him-10* single mutants, because radiation-induced mitotic and meiotic germline defects that occur as a consequence of the *him-10* mutation result in complete sterility. Experiments were repeated twice in each case and error bars correspond to standard deviations. At doses >50 Gy, for which wild type was unaffected, the *him-10* mutant exhibited Late Egg Rad phenotypes other than Gro, although their penetrance was low in comparison with either NHEJ mutants or *him-10*/NHEJ double mutants (data not shown).

However, *mre-11* was crucial for HR-mediated DNA repair in germ cells (Table 1).

For late-stage *C. elegans* embryos where cell division is uniformly arrested for a prolonged period of time, NHEJ mutants were hypersensitive to DNA damage in multiple somatic cell types. Genetic tests excluded the possibilities that *ced-3*- or *cep-1*/p53-mediated apoptosis, *rad-5*/*clk-2*- or *hus-1*-mediated S or G₂/M cell cycle checkpoints, or *daf-12*- or *daf-16*-dependent stress responses might be responsible for induction of the somatic IR-induced phenotypes of late-stage NHEJ mutant embryos (Table 5). Several observations provide a plausible explanation for these phenotypes. First, deficiency for HR did not affect the sensitivity of late-stage NHEJ mutant embryos to IR, indicating that DSBs that occur in late-stage NHEJ mutant embryos are not repaired by HR and may persist as chromosome fragments. Second,

irradiation of late-stage NHEJ mutant embryos resulted in nuclear division failure in intestinal cells of L1/L2 larvae (Figure 8). Third, the spectrum of Late Egg Rad phenotypes is identical to somatic phenotypes previously reported for deficiencies of numerous genes involved in chromosome segregation (O'CONNELL *et al.* 1998; WOOLLARD and HODGKIN 1999; MADDOX *et al.* 2004). Fourth, at low IR doses where chromosome fragments would be expected to be larger and hence often segregate correctly in NHEJ single mutants, double mutants defective for both NHEJ and the *him-10* kinetochore component displayed synergistic postembryonic phenotypes (Figure 9). Taken together, these results suggest that mis-segregation of chromosome fragments is the likely trigger for the somatic developmental abnormalities displayed by irradiated late-stage NHEJ mutant embryos.

A theoretical approach was used to quantitatively assess the prospect that the somatic phenotypes of irradiated late-stage NHEJ mutant embryos might result from mis-segregation of chromosomes. Although *C. elegans* chromosomes are holocentric, studies of free duplications and of microtubule attachment to *C. elegans* chromosomes have suggested that chromosomal fragments smaller than about a tenth of the size of a typical chromosome are likely to mis-segregate, whereas larger chromosome fragments or duplications mis-segregate at lower frequencies (HERMAN *et al.* 1979; ALBERTSON and THOMSON 1982). The *S. cerevisiae* genome has an average of one DSB per cell at a dose of 20 Gy, so the average number of breaks per *S. cerevisiae* diploid cell at a dose of 90 Gy will be 4.5 (LISBY *et al.* 2001). Since *C. elegans* has a genome eight times the size of diploid *S. cerevisiae* and a diploid number of 12 chromosomes, 90 Gy of ionizing radiation may induce $\sim(4.5 \times 8)/12 = 3$ DSBs/worm chromosome during G₁. For a chromosome that has sustained n DSBs, the probability that at least one of the fragments will be less than one-tenth the size of the original chromosome is $p(n) = [1 - (0.8)^n] + [1 - (6/8)^{n-1}] \times (0.8)^n$, where the first term in brackets is the probability that such a break will occur at a distance less than one-tenth of the length of a chromosome from a telomere, whereas the second term in brackets is the probability that if no breaks occur near the telomere, at least two breaks will occur to create a fragment smaller than one-tenth of the size of a chromosome. Assuming a Poisson distribution for the number of breaks with a mean of three breaks per chromosome at 90 Gy, and summing over the number of all possible breaks (n), we find a probability of $P = \sum_n \{\text{Poisson}(n) \times p(n)\} = 0.61$ that any chromosome will have at least one fragment smaller than a tenth of its size, which may mis-segregate if left unrepaired. The probability per nuclear division that at least 1 chromosome of 12 will have a fragment smaller than one-tenth its size is almost 1, and hence almost every postembryonically dividing cell is expected to have at least one

mis-segregation event per cell division. These calculations support the observed synergy of *him-10* with NHEJ mutations (Figure 9) and indicate that the highly penetrant developmental phenotypes of irradiated late-stage NHEJ mutant embryos could result from chromosome mis-segregation.

Studies of vertebrate cells *in vitro* have revealed that NHEJ is used at all stages of the cell cycle, whereas HR is relegated to the late S/G₂-phases (TAKATA *et al.* 1998; ROTHKAMM *et al.* 2003; SALEH-GOHARI and HELLEDAY 2004; HINZ *et al.* 2005). Thus, the radiation hypersensitivity of somatic cells of late-stage *C. elegans* NHEJ mutant embryos might reflect their G₁ arrest. In contrast, noncycling mitotic germ cells rely exclusively on HR-mediated DSB repair, possibly because they arrest in G₂ in late-stage embryos (M. FUKUYAMA and J. ROTHMAN, personal communication) and in dauer larvae. Studies from *S. cerevisiae* and *Schizosaccharomyces pombe* indicate that cell-cycle-dependent fluctuations in the levels of NHEJ are regulated by cyclin-dependent kinase activity rather than by the presence of a sister chromatid (AYLON *et al.* 2004; FERREIRA and COOPER 2004). Specifically, modulation of HR and NHEJ is achieved by cyclin-dependent kinase-mediated regulation of DSB processing at the level of end resection (AYLON *et al.* 2004; IRA *et al.* 2004). Although we lack direct evidence that cell cycle arrest regulates DNA repair in *C. elegans*, the fact that it does so in distantly related species of yeast agrees with our observations regarding developmental modulation of NHEJ. Note that mRNA from the *cku-70*, *cku-80*, and *lig-4* genes is expressed at high levels in the *C. elegans* germline (REINKE *et al.* 2004; Y. KOHARA, personal communication), supporting the possibility that germ cells may repress NHEJ by a mechanism that is distinct from transcriptional regulation.

Stress-resistant dauer larvae may have to store non-dividing germ cells for extended periods of time and may have adapted to this condition by having their germline stem cells arrest in G₂-phase of the cell cycle, where HR-mediated repair is facilitated. Although non-cycling embryonic germ cells arrest for a comparatively brief period of time, G₂ arrest at this additional stage suggests that germ cells may be programmed to arrest in G₂ during all stages of development. Germ cells in *Drosophila* embryos and larvae have also been shown to undergo developmental arrest in the G₂-phase of the cell cycle (SU *et al.* 1998), indicating that this may be an evolutionarily conserved property of germ cells. Although it is presently unclear if arrested embryonic or larval germ cells in *Drosophila* rely on HR for DSB repair, this developmental program may favor HR-mediated DNA repair, thereby helping to ensure error-free DSB repair in cells destined for future generations. It is also possible that G₂ arrest occurs for other reasons pertinent to germ cell biology (ASAOKA-TAGUCHI *et al.* 1999). We speculate that the coupling of germ cell identity with a cell-cycle-regulated DNA repair program

may have originated in the first multicellular organisms that contained distinct germ and somatic cell lineages, perhaps offering a selective advantage to germ cells during diapause, an arrested developmental stage that is relevant to the life cycles of many organisms. Although human female germ cells can arrest in the first meiotic prophase for decades, prepubescent human male germ cells arrest for many years in G₁. The stage at which male germ cells arrest during development remains an open question for *C. elegans*, and it is possible that this trait may be sexually dimorphic. This study demonstrates the utility of examining DNA repair in the context of a whole organism and suggests that developmental modulation of NHEJ or NHEJ-mediated processes, such as the restriction of V(D)J recombination to G₁ in the mammalian lymphatic system, may have evolved several times. Our findings may be relevant to the development or treatment of cancers that originate from either germ or somatic cells.

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