The Drosophila *chk2* Gene *loki* Is Essential for Embryonic DNA Double-Strand-Break Checkpoints Induced in S Phase or G2

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ABSTRACT

Cell cycle checkpoints are signal transduction pathways that control the order and timing of cell cycle transitions, ensuring that critical events are completed before the occurrence of the next cell cycle transition. The Chk2 family of kinases is known to play a central role in mediating the cellular responses to DNA damage or DNA replication blocks in various organisms. Here we show through a phylogenetic study that the *Drosophila melanogaster* serine/threonine kinase Loki is the homolog of the yeast Mek1p, Rad53p, Dun1p, and Cds1 proteins as well as the human Chk2. Functional analyses allowed us to conclude that, in flies, *chk2* is involved in monitoring double-strand breaks (DSBs) caused by irradiation during S and G2 phases. In this process it plays an essential role in inducing a cell cycle arrest in embryonic cells. Our results also show that, in contrast to *C. elegans chk2*, Drosophila *chk2* is not essential for normal meiosis and recombination, and it also appears to be dispensable for the MMS-induced DNA damage checkpoint and the HU-induced DNA replication checkpoint during larval development. In addition, Drosophila *chk2* does not act at the same cell cycle phases as its yeast homologs, but seems rather to be involved in a pathway similar to the mammalian one, which involves signaling through the ATM/Chk2 pathway in response to genotoxic insults. As mutations in human *chk2* were linked to several cancers, these similarities point to the usefulness of the Drosophila model system.

SURVEILLANCE pathways called cell cycle checkpoints are in place to ensure that cells replicate the genetic information with high fidelity and that the chromosomes are properly passed on to the daughter cells (Abraham 2001). The components of the checkpoint pathway are highly conserved from yeast to human and the pathway can be divided into four parts: initiating signals, sensors, transducers, and effectors (Elledge 1996). In response to stress signals sensed during different phases, cells can induce cell cycle arrest in G1, S, G2, or M phase, activate DNA repair pathways and transcription programs, regulate telomere length, and induce apoptosis (Elledge 1996; Ritchie *et al.* 1999; Hirao *et al.* 2000). These cell cycle checkpoints are signal transduction pathways that control the order and timing of cell cycle transitions. Checkpoint defects therefore result in genomic instability and have been associated with cancer predisposition (Hartwell and Kastan 1994; Bell *et al.* 1999).

During Drosophila larval development, DNA damage checkpoint pathways also function as an inducible DNA damage/replication checkpoint (Sibon *et al.* 1999; Brodsky *et al.* 2000; Xu *et al.* 2001; Peters *et al.* 2002). Imaginal disc cells contain a typical cell cycle with G1, S, G2, and M phases; in these cells, irradiation activates the DNA damage checkpoint that arrests cells at the G2/M transition and induces p53-mediated apoptosis. *mei-41*, *grapes*, *mus-304*, *14-3-3ε*, and *loki/chk2* are required for this checkpoint response.

In the mammalian system, Chk2 is a key player in maintaining the genome integrity. In the G1 checkpoint, ionizing radiation (IR) exposure can activate the ataxia-telangiectasia-mutated (ATM)-Chk2 pathway. Activated Chk2 phosphorylates Ser123 in Cdc25A, targeting it for ubiquitin-dependent degradation (Falck *et al.* 2001). As Cdc25A is downregulated, the activity of CyclinA-Cdk2 is inhibited and replication is slowed down. In addition, downregulation of Cdc25A is thought to inhibit the activity of CyclinE-Cdk2, leading to the p53-independent initiation of the G1 checkpoint (Bartek and Lukas 2001). In the G2/M checkpoint, Chk1, Chk2, and p53 are three key transducers: the *A*TM and *R*ad 3-related (ATR)-Chk1 pathway is thought to be activated when cells are exposed to IR during G1 or S phase; the ATM-Chk2 pathway is thought to arrest cells in response to genotoxic insults during G2 phase (Abraham 2001); and recent findings indicate that p53 may play additional roles to help cells arrest at the G2/M transition (Taylor and Stark 2001). Checkpoint signals relayed from Chk1, Chk2, and p53 arrest the cell cycle at the G2/M transition via downregulation of the kinase activ-

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ity of CyclinB-Cdk1. Depending on the transducer, this downregulation step can involve one of the following intermediates: Cdc25C (Peng *et al.* 1997; Brown *et al.* 1999), p21 (Boulaire *et al.* 2000; Smits *et al.* 2000), Gadd45 (Zhan *et al.* 1999), or 14-3-3σ (Taylor and Stark 2001).

While most checkpoint genes are nonessential genes under laboratory conditions, some are essential for cell viability and survival of the organism (Brown and Baltimore 2000; Takai *et al.* 2000). This suggests that checkpoint pathways are not only surveyors of occasional damage, but also firmly integrated components of cellular physiology (Zhou and Elledge 2000). In fact, in Drosophila, the checkpoint pathway acting through *mei-41* (the ATR ortholog) and *grapes* (*grp*, the Drosophila *chk-1*) is also used in a developmental context to slow down the cell cycle and to control gene expression, and its disruption causes defects in pattern formation (Fogarty *et al.* 1994; Sibon *et al.* 1997, 1999; Ghabrial and Schüpbach 1999; Brodsky *et al.* 2000).

However, checkpoints are not exclusive to cells that divide mitotically; they also operate during meiosis since homologous recombination occurs ~100–1000 times more frequently during meiotic prophase than during mitosis (Fox and Smith 1998). With this important difference, it is not surprising that meiotic cells developed a meiotic-specific checkpoint control in addition to the normal DNA damage/replication and spindle checkpoints. This pachytene or meiotic recombination checkpoint prevents exit from the pachytene stage of meiotic prophase I when meiotic recombination and chromosome synapsis are incomplete (Lydall *et al.* 1996).

In budding yeast, Mek1p is highly homologous to Rad53p and is thought to function in the pachytene checkpoint pathway as a counterpart of Rad53p in the mitotic DNA damage checkpoint pathway. Recombination causes the formation of single-strand DNA in the recombination intermediates and, as a response, Mec1p phosphorylates and activates Mek1p, which, in turn, phosphorylates Red1p, a major component of the axial element of meiotic chromosomes (Smith and Roeder 1997; de los Santos and Hollingsworth 1999). The pachytene arrest is achieved by inhibiting the cyclin-dependent kinase complex, Clb1p-Cdc28p (Chu and Herskowitz 1998). Once recombination is completed successfully, the inhibitory signal is eliminated by Glc7p-dependent dephosphorylation of Red1p, allowing progression toward the pachytene exit.

Drosophila females mutant for genes involved in the meiotic pachytene checkpoint, such as *mei-41* and *mus304*, show a high chromosome nondisjunction rate and a low recombination frequency, indicating a defect in the meiotic recombination checkpoint (Ghabrial *et al.* 1998; Sibon *et al.* 1999; Brodsky *et al.* 2000). *okr*, *spn-B* (orthologs of *Saccharomyces cerevisiae* Rad54 and Dmc1, respectively), and *spn-C* mutations also cause the accumulation of double-strand breaks (DSBs) and a continuously activated pachytene checkpoint that arrests oocytes in prophase I. The ensuing patterning defects seem to result from this meiotic arrest as they can be suppressed with a mutation in the checkpoint kinase *mei-41* (Ghabrial *et al.* 1998; Ghabrial and Schüpbach 1999). Furthermore, alleles of the *spn* genes demonstrate mislocalization of *grk* mRNA as well as defects in post-translational modification of the Vasa protein. Because Vasa is required for the normal accumulation of Grk in the oocyte (Styhler *et al.* 1998; Tomancak *et al.* 1998), these observations suggest that the detection of DSBs activates a meiotic checkpoint that inhibits Vasa from inducing the accumulation of Grk in the anterior region of the oocyte.

Recently, genetic analysis of *Caenorhabditis elegans chk2* revealed another function in meiosis. This gene is required during the initial phase of meiosis for the establishment of homologous pairing. Its function as a checkpoint transducer, however, is not clear yet (MacQueen and Villeneuve 2001).

Here we show that *loki/chk2* is an essential DNA double-strand-break checkpoint component that acts during the embryonic S and G2 phases. We also show that *loki*/chk2 is dispensable for normal development as well as for DNA synthesis, recombination, and other DNA damage checkpoints.

MATERIALS AND METHODS

Generation of *loki*^{null} mutants: To create a *loki*^{null} mutant, we mobilized a *P* element that inserted into the 5′ region of the *barren* gene [*l(2)k08103*; Bhat *et al.* 1996]. *Df(2L)be408*, a deficiency that affects the genomic sequences of *loki* and its two neighboring genes, *barren* and *CG10728*, was thereby isolated. *Df(2L)pr2b*, a larger deficiency that also removes *loki*, was also used. To restore the function of *barren*, the rescue construct *P[w*$^+$*, barren*$^+$*]* was reintroduced into the *Df(2L)pr2b* chromosome using *P*-element-mediated transformation (Figure 1). In the same way, the *P[w*$^+$*, CG10728*$^+$*]* insertion was reintroduced into the *Df(2L)be408* chromosome. *loki*^{null} (*chk2*^{null}) females are *w/w; Df(2L)be408, P[w*$^+$*, CG10728*$^+$*]/Df(2L)pr2b, P[w*$^+$*, barren*$^+$*]*.

Phylogenetic analysis: Protein sequences were obtained from the National Center for Biotechnology Information (NCBI) databases (http://www.ncbi.nlm.nih.gov/). Loki's homologs were identified with Blast-Protein searches in the nonredundant database (Altschul and Lipman 1990). Multiple alignments using CLUSTAL X (Thompson *et al.* 1994) were performed to determine the most conserved region. The alignment was then checked and optimized manually using the published kinase alignment as a guide (Hanks and Hunter 1995). This modified alignment was then used for phylogenetic analysis. Unrooted phylogenetic trees were derived from neighbor-joining approaches in CLUSTAL X and 100 bootstrap replicates were generated using the same program.

Generation of an antibody against Loki: The N-terminal part of the *loki* cDNA [0–1060 bp, 0–263 amino acids (aa)] was cloned into the pQE expression vector (QIAGEN, Chatsworth, CA) to make a His-tag fusion protein. The induced fusion protein was purified first against a Ni-NTA matrix and then

through SDS-PAGE electrophoresis. The Loki protein band was excised from the gel, eluted in electrophoresis buffer, and injected into rabbits. The immune serum was affinity purified against full-length Loki-MBP conjugated to a Sepharose 4B column (Amersham Pharmacia Biotech).

Western blotting: Ovary and embryo extracts were prepared by dissecting ovaries in Ringer's buffer and freezing them immediately on dry ice. Frozen ovaries were homogenized in 2× SDS loading buffer (5 μl/ovary) on ice and boiled for 10 min. Embryos were collected and aged on apple juice plates at 25°. Aged embryos were washed, dried, and weighed. Dried embryos were homogenized in 2× SDS loading buffer (100 μg/μl) on ice, boiled for 10 min, and centrifuged to get the supernatants. Protein samples were resolved on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes, and probed for Loki. Rabbit anti-Loki serum was used at a dilution of 1:2000–5000. As a loading control, blots were probed for α-tubulin with the monoclonal antibody (DM1α; Sigma, St. Louis) at a dilution of 1:500. Horseradish-peroxidase-conjugated anti-rabbit IgG was used at a dilution of 1:2000. Luminol (Amersham Pharmacia Biotech) staining was done for 1 min. A phosphorimager (Molecular Dynamics, Sunnyvale, CA) was used to determine quantitative levels of proteins.

Meiotic nondisjunction test: To assess the frequency of nondisjunction in *loki* mutant females, *+/Y* males were crossed to *w/w; Df(2L)be408, P[w$^+$, CG 107278$^+$]/Df(2L)pr2b, P[w$^+$,barren$^+$]*. A cross of *+/Y* males to *w/w; Df(2L)be408, P[w$^+$, CG 107278 $^+$]/CyO* was used as a control. Both male and female progeny were scored. Exceptional *w/w/Y* females can be distinguished from their *w/+* sisters, and exceptional *+/0* males can be distinguished from their *w/Y* brothers.

MMS/HU sensitivity tests: A total of 5–10 groups, each containing five virgins and five males of appropriate genotypes, were crossed. The resulting embryos were collected for 24 or 48 hr and the adult flies were then removed. After an additional 24 hr at 25°, 250 μl of 0.08% methyl methanesulfonate (MMS; Sigma, 64382), dissolved in distilled water, or 20 mM hydroxyurea (HU; Sigma, H8627) was added to each vial. After 2 weeks, all classes of adult progeny were scored. MMS sensitivity is indicated by the preferential loss of a specific genotypic class relative to the control.

Embryo irradiation: Fly embryos were collected on apple juice plates at 25° and aged to reach the desired cell cycle stages. Apple juice plates containing staged embryos were irradiated at 4930 rad/hr in a gamma-cell 40 low-dose-rate laboratory irradiator (Atomic Energy of Canada).

Antibody staining and pole cell counting: Embryos were staged according to Bownes (Bate and Arias 1993), dechorionated with 50% bleach, washed with tap water, and fixed for 20 min in a mixture (1:1) of heptane and PBS containing 4% paraformaldehyde. Fixed embryos were devitellinized in a mix (1:1) of heptane and methanol/EGTA (90% methanol; 10% 0.5 M EGTA, pH 8.0) for 10–20 min. Devitellinized embryos were washed with methanol/EGTA two to three times and blocked in PBST + 0.1% BSA + 5% goat serum for 3–4 hr (with changes of buffer in between) before proceeding with the antibody staining. Rabbit polyclonal anti-phospho-histone H3 (PH3) antibody (06-570; Upstate Biotechnologies, Lake Placid, NY) was used at a dilution of 1:500; rat polyclonal anti-Vasa antibody was used at a dilution of 1:2000. Secondary antibodies were Cy3-conjugated anti-rabbit and Oregon-green488-conjugated anti-rat antibodies. Stained embryos were observed under a Zeiss Axioplan microscope and pictures were taken with a SPOT camera (DIAGNOSTIC).

Similarly, rabbit polyclonal anti-Vasa (1:5000) and biotinylated anti-rabbit IgG secondary antibody were used to stain 3- to 4-hr-old embryos to count the pole cells. The Vectastain kit (Vector Laboratories, Burlingame, CA) was then used for the signal enhancement treatment: after the final wash step, embryos were incubated for 1 hr at room temperature in the ABC solution. They were then incubated in a mixture of 4 mg/ml diaminobenzine solution and PBS (1:9) for 5 min, after which 2 μl of H_2O_2 (1:100 of a 30% stock) was added. The progress of the staining reaction was followed under the microscope and stopped by rinsing with PBST three times for 10 min. Embryos were then dehydrated by consecutive washing in 25, 50, 75, 95, and 100% ethanol in PBS and incubated overnight in Histoclear at 4°. They were finally mounted in Permount and left with a weight on the coverslip overnight.

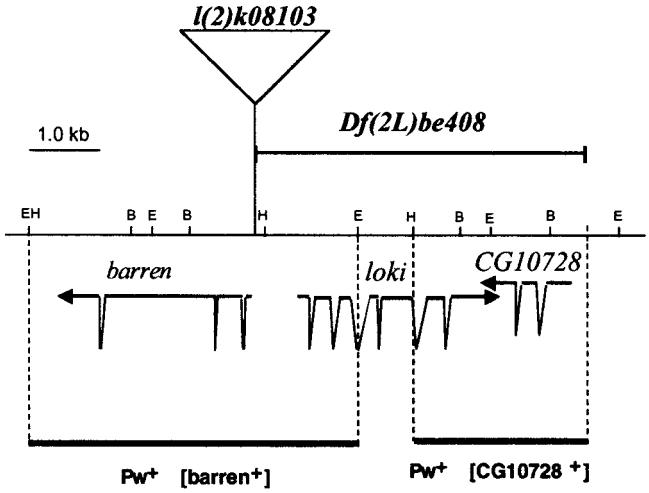

Figure 1.—Genomic organization of the *loki* region showing the location of the *P* element used to create the *loki^{null}* mutant [*Df(2L)be408*] and the extent of the rescue constructs introduced for the flanking genes.

RESULTS

Phylogenetic analysis of Loki and its homologs: The *loki* gene (Figure 1) was identified in a screen for serine/threonine protein kinases that are expressed in the Drosophila ovary. Sequenced PCR fragments were used to screen genomic and cDNA libraries (Larochelle and Suter 1995). The cDNA sequence has the GenBank accession no. 004364.

We then performed a phylogenetic analysis with Loki and its most similar sequences. First, the Loki polypeptide sequence identified 48 sequences with considerable identity (>25%) in the NCBI databases using the BLAST algorithm. The most conserved sequence, the kinase domain, was then used to perform a multiple alignment that served to generate the phylogenetic tree. The neighbor-joining phylogeny produced a high percentage (94%) branched clade containing Loki, Chk2, Mek1p, Rad53p, Cds1, and Dun1p (Figure 2). In addition, Loki contains a FHA domain (52–112 aa) followed by a kinase domain (157–424 aa), which is the distinguishing feature of Rad53p, Mek1p, Dun1p, Cds1, and Chk2. It is thus likely to have a similar checkpoint function in flies as its homologs in their respective organ-

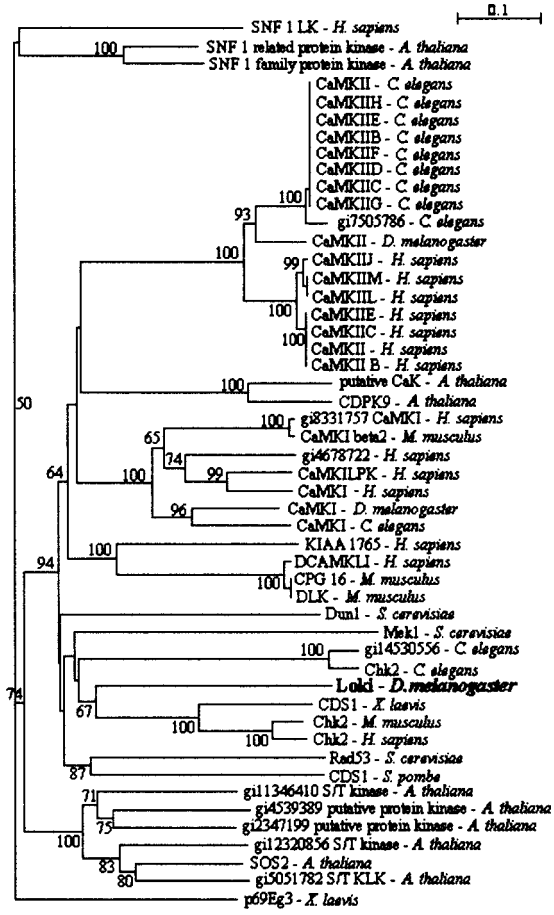

FIGURE 2.—Neighbor-joining phylogeny of Loki and its homologs in *Arabidopsis thaliana*, *S. cerevisiae*, *S. pombe*, *C. elegans*, *D. melanogaster*, *X. laevis*, *M. musculus*, and *H. sapiens* using the kinase domain alignment. Loki is shown in boldface type; unnamed proteins begin with "gi" numbers. Branch lengths are proportional to the estimated numbers of amino acid substitutions per site. Bootstrap values >50% are given at branch nodes.

isms. Because of its phylogenetic position and because of its checkpoint function (see below), we call it Chk2 in the remainder of this article.

***chk2* and germline establishment:** OISHI *et al.* (1998) reported that both *chk2* transcripts and polypeptides are transiently present throughout the embryo at the early syncytial stages, but become restricted to the posterior pole and then to the pole cells (primordial germ cells) by the blastoderm stage where they perdure during gastrulation. On the basis of the expression data above, they proposed that *chk2* might play a role in germline establishment and/or maintenance.

We made a $chk2^{null}$ stock that can be maintained over generations, indicating that *chk2* has no essential role in germline establishment. Pole cells cease dividing after the formation of the cellular blastoderm. They arrest in the G2 phase of the cell cycle until they coalesce with the somatic gonadal precursor cells to form the primitive gonad. To assess the potential involvement of *chk2* in greater detail, we compared the number of migrating pole cells in embryos from $chk2^{null}$ mothers to wild type and found an average of 24.5 pole cells/embryo ($n = 30$) in the former compared to 37.3 ($n = 33$) in the latter. It therefore seems that *chk2* plays a role in the maintenance of the germline, although we cannot exclude the possibility that reduced levels of expression from the transgenes containing $barren^{+}$ or $CG10728^{+}$ are insufficient for normal germ cell maintenance.

***chk2* is not required during normal meiosis:** Under normal laboratory conditions $chk2^{null}$ mutant flies produced viable and fertile progeny. Even though the $chk2^{null}$ stock does not grow as well as the Oregon-R stock, this still indicates that *chk2* is a nonessential gene that is also not essential for fertility. We next tested whether *chk2*, like its yeast and *C. elegans* homologs, is required for normal recombination and chromosome segregation. In crosses that allowed us to score the exceptional females and males produced by X chromosome nondisjunction in the F_1 progeny, we observed no increase in the rate of X chromosome nondisjunction between $chk2^{null}$ mutants (0/2504) and $chk2^{-/+}$ heterozygous flies (0/1614). Furthermore, females lacking *chk2* displayed no defect in meiotic recombination frequency (J. VELEMA and B. SUTER, personal communication). In contrast to *C. elegans*, these results mean that *chk2* is not an essential element of the meiotic checkpoint in Drosophila.

In addition, pattern formation has been linked to the activation of a *mei-41*-dependent meiotic checkpoint pathway. This pathway is activated in response to unrepaired DSBs during meiosis as they accumulate in several *spn* mutants (GHABRIAL and SCHÜPBACH 1999). To check if *chk2* is part of this checkpoint, we generated *chk2*; *spn* double mutants and tested whether the dorsoventral patterning defects found in *spnB* and *spnC* mutant eggs could be rescued by the removal of *chk2*. Eggs laid by *chk2; spnB* and *chk2*; $spnC^{094}$ double-mutant females were classified as wild type, fused, or missing dorsal appendages. *spn* mutant sisters that have one wild-type copy of *chk2* were used as internal controls. Furthermore, we also generated *mei-41; spn* and *grp; spn* double mutants. As shown by GHABRIAL and SCHÜPBACH (1999), removal of *mei-41* rescued the ventralized egg shell phenotype of *spn* mutants; however, removal of neither *chk2* nor *grp (chk1)* rescued the phenotype (Table 1). It thus appears that this *mei-41*-dependent meiotic checkpoint signal is transduced by either a redundant pathway or a *chk2*-independent mechanism.

***chk2* is not required for the larval DNA replication checkpoint or for the DNA damage checkpoint activated by alkylating agents:** To test whether *chk2* functions during larval stages in either of these checkpoints, we analyzed the sensitivity of $chk2^{null}$ larvae to MMS, a DNA damage reagent, and to HU, a DNA replication blocking

TABLE 1

Dorso-ventral patterning of the egg shell in *spn* mutants: *mei-41; spn* and *chk2; spn* double mutants

Maternal genotype	Wild-type dorsal appendages (%)	Fused dorsal appendages (%)	No dorsal appendages (%)
$spnC^{094}/spnC^{094}$ (control)	23/90 (26)	45/90 (50)	22/90 (24)
$mei\text{-}41^{RT1}\ f^{1}/mei\text{-}41^{D3}$; $spnC^{094}/spnC^{094}$	78/111 (70)	23/111 (21)	10/111 (9)
$mei\text{-}41^{RT1}\ f^{1}$/FM7c; spnB/spnB (control)	37/105 (35)	20/105 (19)	48/105 (46)
$mei\text{-}41^{RT1}\ f^{1}/\ mei\text{-}41^{D3}$; spnB/spnB	87/134 (65)	34/134 (25)	13/134 (10)
Df(2L)be408, P[w+, CG10728^{+}] or Df(2L)pr2b, P[w+, barren^{+}]/CyO; $spnC^{094}/spnC^{094}$ (control)	30/175 (17.1)	87/175 (49.7)	58/175 (33.1)
Df(2L)be408; P[w+, CG10728^{+}]/ Df(2L)p2b, P[w+, barren^{+}]; $spnC^{094}/spnC^{094}$	29/160 (18.1)	85/160 (53.1)	46/160 (28.75)
Df(2L)be408, P[w+, CG10728^{+}] or Df(2L)pr2b, P[w+, barren^{+}]/CyO; spnB/spnB (control)	37/150 (24.6)	13/150 (8.6)	100/150 (66.7)
Df(2L)be408, P[w+, CG10728^{+}]/ Df(2L)pr2b, P[w+, barren^{+}]; spnB/spnB	37/156 (23.7)	17/156 (10.9)	102/156 (65.4)
grp^{fsA4}/CyO; spnB/spnB (control)	35/110 (31.8)	28/110 (25.4)	47/110 (42.7)
grp^{fsA4}/grp^{fsA4}; spnB/spnB	33/95 (34.7)	26/95 (27.4)	36/95 (37.9)

reagent. In this assay, *grp* mutant flies were used as a positive control, and $chk2^{null}$ and $grp^{fs(A)4}$ homozygous larvae were generated independently and raised on food supplied with low levels of either HU (20 mM) or MMS (0.08%). Sensitivity is indicated by a preferential loss of homozygous offspring in the presence of MMS and HU. By crossing balanced heterozygous females to balanced heterozygous males, homozygous mutants:heterozygous mutants:Balancer/Balancer offspring are produced at the ratio 1:2:1. Because Balancer/Balancer flies die before they can be scored, a ratio of 1:2 for homozygous:heterozygous mutants would indicate that the mutant flies are fully resistant to the treatment. Consistent with published results (Sibon *et al.* 1999), *grp* mutants were sensitive to MMS (the ratio of homozygous:heterozygous flies in the F_1 progeny is 0:288) and to HU (0:113). However, no loss of $chk2^{null}$ mutant flies was observed after treatment with either MMS (the ratio of homozygous:heterozygous flies in the F_1 progeny is 90:189) or HU (91:174), indicating that *chk2* plays no essential function in these checkpoint pathways during larval development. In contrast to these results, *chk2* was found to function in inducing cell cycle arrest and *p53*-mediated apoptosis upon the activation of a DNA double-strand-break checkpoint in the larval imaginal discs (Xu *et al.* 2001; Peters *et al.* 2002).

***chk2* expression:** Northern hybridization with the *chk2* cDNA revealed that the expression of its RNA is temporally restricted during development (Figure 3A). The *chk2* RNA appears to accumulate primarily in the ovary and in embryos during the first 2 hr of development, indicating that *chk2* RNA is maternally deposited into the egg. Although the *chk2* transcript signal is not spatially restricted to any part of the young embryos (Oishi *et al.* 1998), in ovaries, *chk2* mRNA is first detected in region 2 of the germarium, where it concentrates in the prospective oocyte. Later it is clearly found to accumulate in the oocyte, at the posterior end up to stage 7. From stage 8 to 10, it is found at the anterior cortex of the oocyte (Figure 3B).

A polyclonal antibody raised against Chk2 recognizes a specific band of ~52 kD in wild-type embryos but not in embryos from *chk2* mutant mothers (Figure 4). Even though *chk2* mRNA accumulates at high levels during oogenesis (Figure 3A), the protein is hardly detectable in this tissue (Figure 4). However, a strong Chk2 signal is found during the first 4 hr of embryonic stages (0–4 hr after egg deposition). After that, the Chk2 signal drops again to background levels. Since the Chk2 protein expression suggests that the gene may function during early embryogenesis, we wanted to determine whether *chk2* functions in DNA damage checkpoint activation in 3- to 4-hr-old embryos, which are in cycle 14.

***chk2* is required for the embryonic DNA damage checkpoint activation induced by γ-ray irradiation:** During cycle 14, cells are known to enter mitosis as stereotypical clusters called "mitotic domains." The timing of entry into mitosis of each one of these domains as well as the morphogenetic movements that comprise gastrulation are known to be invariant between different embryos (Foe 1989). It was furthermore observed that DNA damage induced by irradiation or MMS treatment can

delay entry into mitosis of cycle 14; this delay is primarily due to inhibitory phosphorylation of Cdk1, and nuclear exclusion of the Cdk1-Cyclin complex might also play a secondary role (Su *et al.* 2000).

We exposed 130- to 200-min-old embryos (during which cells are in interphase 14) to 600 rad of γ-irradiation, which corresponds to the half-lethal dose. Irradiated embryos were allowed to recover for 45 min, after which they were fixed and stained for the mitotic-specific PH3 epitope. At the same time these embryos were stained for Vasa, a pole-cell-specific marker that shows the progression of the morphogenetic movements of gastrulation. In nonirradiated wild-type embryos, domain 1 initiated mitosis in stage 6 (Figure 5A1). In wild-type embryos, irradiation caused a delay of entry into mitosis. For instance, domain 1 did not start mitosis until much later after irradiation (stage 8; Figure 5B3). In nonirradiated *chk2*^{null} mutant embryos, mitotic patterns in each specific gastrulation stage were the same as in the nonirradiated wild-type embryos (Figure 5, A1–4 and C1–4). However, in irradiated *chk2*^{null} mutant embryos, each mitotic domain entered mitosis with the same timing as the nonirradiated control, indicating that the DNA damage checkpoint was defective. For example, in embryonic stage 8, PH3 staining showed the presence of mitotic domains 1–6 in both nonirradiated and irradiated embryos (Figure 5, C3 and D3). Similar defects in arresting the cell cycle were observed in irradiated *chk2*^{null} embryos that were allowed to recover for only 15 min after the γ-ray exposure (data not shown). As S and G2 phases of cycle 14 last 50 and 20 min, respectively (Foe 1989), this finding shows that *chk2* is a damage checkpoint gene involved in mediating responses to DSBs induced during the S or G2 phases of the cell cycle.

Oishi *et al.* (1998) reported that the Chk2 polypeptide does not accumulate in the somatic tissue after cellularization. However, our results show that *chk2* is required for the DNA damage checkpoint in these somatic cells. It thus appears that low Chk2 levels are sufficient for this checkpoint to be active.

***orb* represses Chk2 expression in the ovary:** The fact that Chk2 polypeptide expression is very low in the ovary despite the high mRNA levels (Oishi *et al.* 1998; Figures 3 and 4) may suggest that *chk2* mRNA is translationally repressed during oogenesis. UV crosslinking experiments showed that a polypeptide with the mobility of Orb binds to the *chk2* 3′ untranslated region (A. Nakamura, personal communication). To test whether *orb* is responsible for the low levels of ovarian Chk2 polypep-

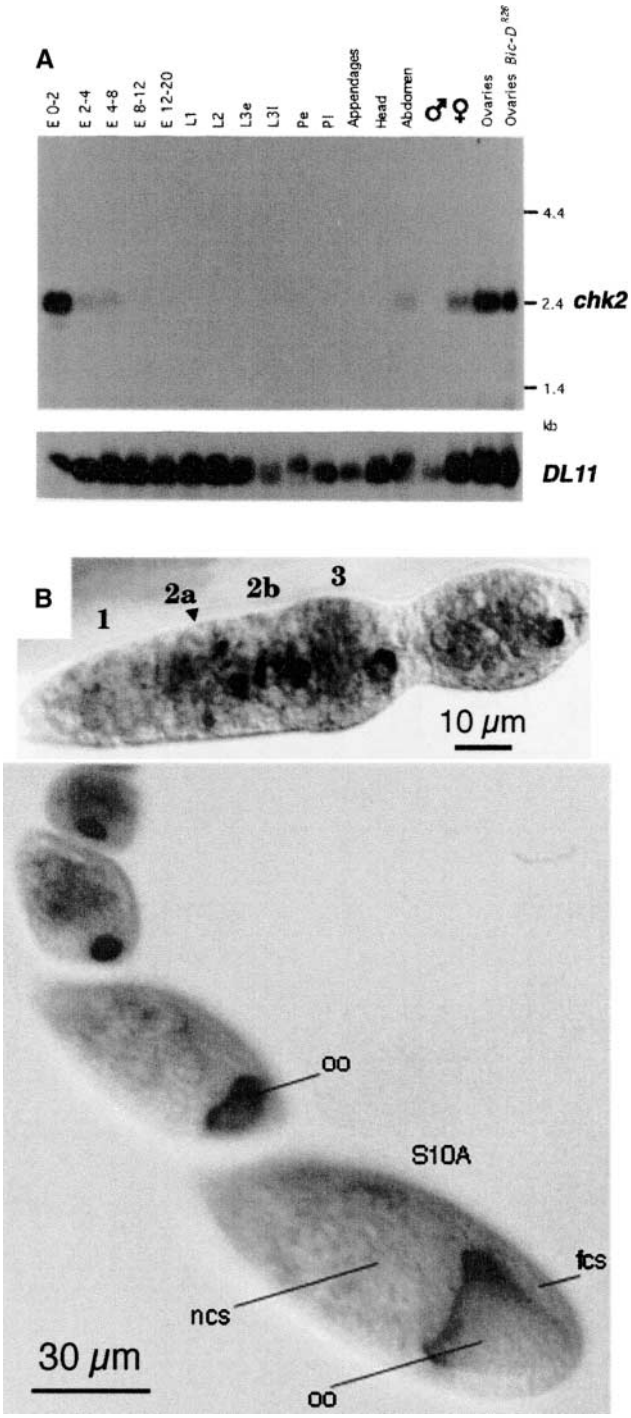

Figure 3.—Expression of *chk2* transcripts. (A) Developmental Northern blot. Embryonic stages (E) are in hours after egg deposition: L1, first instar larvae; L2, second instar larvae; L3e, early third instar larvae; L3l, late third instar larvae; Pe, early pupae; Pl, late pupae. *chk2* is a single mRNA species of 2.4 kb that is present in females, ovaries, and early embryos. It is also present in *BicD*^{R26} ovaries that fail to make an oocyte. An autoradiograph of the same filter after hybridization with the small ribosomal subunit protein gene L11 shows the amount of mRNA loading. (B) Wild-type *chk2* mRNA distribution patterns during oogenesis. Some oocytes (oo), nurse cells (ncs), and follicle cells (fcs) are indicated for clarity. In the germarium (top), *chk2* transcripts localize to the prospective oocyte as early as germarial region 2b.They remain localized to the oocyte during early oogenesis. After stage 9, *chk2* mRNA acquires an anterior localization pattern and accumulates at the oocyte/nurse cell interface.

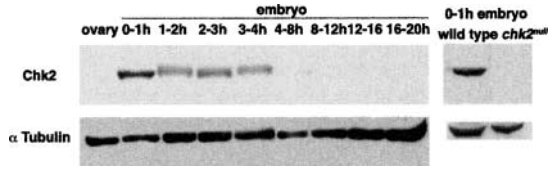

FIGURE 4.—Expression of Chk2 protein. Western blot shows Chk2 protein levels in wild-type ovary and embryo extracts of different stages. α-Tubulin serves as a loading control.

tide, *trans*-heterozygous females of different combinations of the *orb* alleles orb^{F343}, orb^{F303}, and orb^{mel} were analyzed for their ovarian Chk2 levels. orb^{F343} is a strong loss-of-function allele, orb^{F303} is slightly less severe than orb^{F343}, and orb^{mel} is a weak allele (CHRISTERSON and MCKEARIN 1994; LANTZ *et al.* 1994). Quantitative analysis of the Western blot, using eIF4A protein levels as reference, shows that Chk2 polypeptide levels are almost 10-fold higher in *orb* mutant ovaries than in wild-type ones (Figure 6).

One of the *orb* phenotypes is a defect in dorso-ventral patterning of the egg shell (CHRISTERSON and MCKEARIN 1994; LANTZ *et al.* 1994). This phenotype can also be caused by the activation of a meiotic checkpoint triggered by unrepaired DNA double-strand breaks (GHABRIAL and SCHÜPBACH 1999). To test whether *orb* mutations cause a dorso-ventral defect through the over-accumulation of the checkpoint kinase Chk2, we generated *chk2; orb* double-mutant flies. Double-mutant females were mated to wild-type males and the laid eggs were classified as nonfused, fused, or missing dorsal appendages (Table 2). As internal controls, *orb* mutant sisters from the same cross were used; these females had one wild-type copy of *chk2*. As shown in Table 2, removing *chk2* does not clearly rescue the ventralized egg shell phenotype of *orb*, indicating that *orb* mutations cause this defect through another pathway. It thus seems that high levels of Chk2 alone do not induce its cellular function. This is in fact consistent with results from Chk2 orthologs, which show that these kinases need to be phosphorylated to be activated (BARTEK *et al.* 2001).

DISCUSSION

Mutations in human *chk2* have been linked to some cases of Li-Fraumeni syndrome, characterized by a predisposition to cancer (BELL *et al.* 1999). The *CHEK2*11-00delC* variant was also found associated with an ~2-fold (in women) and 10-fold (in men) increase of breast cancer risk in noncarriers of BRCA1 or BRCA2 muta-

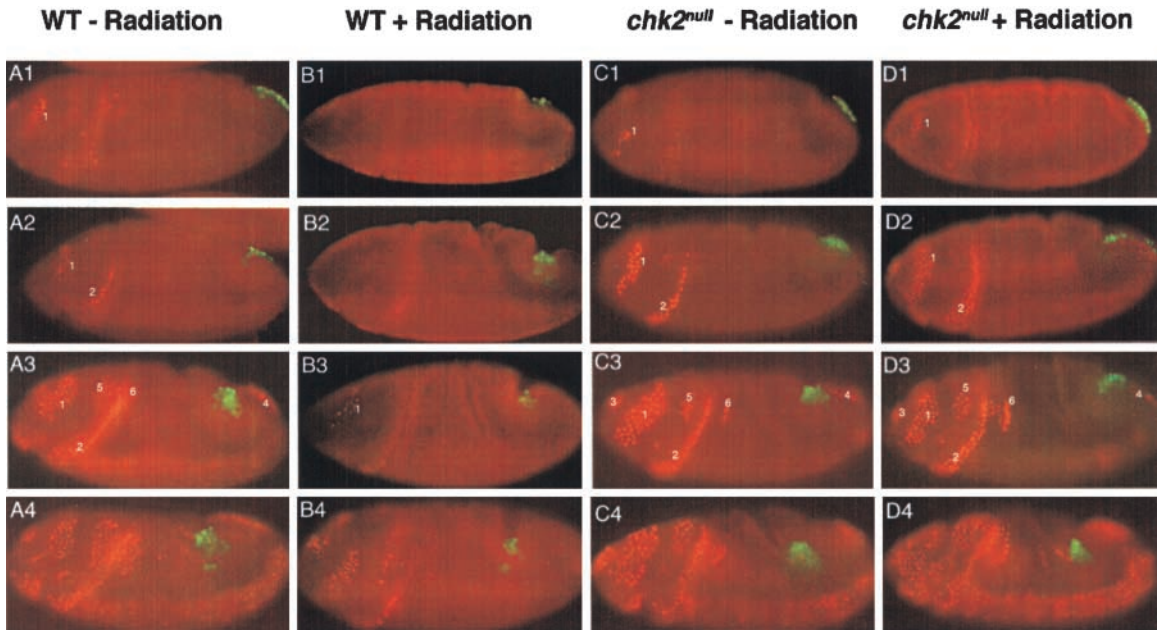

FIGURE 5.—The effect of γ-irradiation on wild-type and *chk2* mutant embryos (mutant refers to the maternal genotype). PH3 staining is shown in red to show mitotic domains and Vasa staining (green) shows the progression of gastrulation. The numbers 1–4 indicate different gastrulation stages from early to late. A1–4 are wild-type embryos without irradiation. B1–4 are wild-type embryos fixed 45 min after irradiation with 600 rad γ-rays. C1–4 are *chk2* mutant embryos without irradiation. D1–4 are *chk2* mutant embryos fixed 45 min after irradiation with 600 rad γ-rays. Mitotic domains 1–6 are shown in A1–3, B1–3, C1–3, and D1–3. In wild-type embryos, irradiation caused a delay of entry into mitosis. For instance, domain 1 did not start mitosis until much later after irradiation (stage 8; B3). In nonirradiated $chk2^{null}$ mutant embryos, mitotic patterns in each specific gastrulation stage were the same as those in the nonirradiated wild-type embryos (A1–4 and C1–4). However, in irradiated $chk2^{null}$ mutant embryos, each mitotic domain entered mitosis with the same timing as the nonirradiated control, indicating that the DNA damage checkpoint was defective. For example, in embryonic stage 8, PH3 staining showed the presence of mitotic domains 1–6 in both nonirradiated and irradiated embryos (C3 and D3).

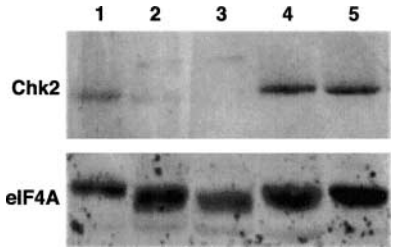

FIGURE 6.—Repression of Chk2 expression by *orb*. (1) Wild-type embryo extracts; (2) wild-type ovary extracts; (3) $chk2^{null}$ ovary extracts; (4) orb^{303}/orb^{mel} ovary extracts; and (5) orb^{343}/orb^{mel} ovary extracts.

tions (MEIJERS-HEIJBOER *et al.* 2002). A good invertebrate model for *chk2* can hence be very interesting from a medical point of view. The phylogenetic tree, based on the kinase domain alignment, showed with a high probability (94%) that the *Drosophila melanogaster* Chk2 is a member of a clade containing Chk2 (*C. elegans*, *Mus musculus*, and *Homo sapiens*), a *C. elegans* Chk2 closely related protein (gi14530556), Cds1 (*Schizosaccharomyces pombe* and *Xenopus laevis*), Mek1p, Rad53p, and Dun1p (*S. cerevisae*). This close evolutionary relationship suggests a functional similarity. In contrast to this, the Arabidopsis putative protein kinase is in a different clade, even though the BLAST search revealed that the Drosophila Chk2 has the highest homology to this kinase over the entire peptide sequence. Furthermore, Dun1p branches off early from the Chk2 family clade even though it is also a checkpoint signaling kinase that contains a FHA domain. The position in the tree correlates with the role Dun1p plays in the DNA damage and replication checkpoint pathways, because, unlike Rad53p, Dun1p functions in a parallel pathway, not to arrest the cell cycle but, as a transcriptional activator, to activate the expression of the DNA repair genes (ELLEDGE 1996).

In *S. cerevisiae*, the Chk2 family member Rad53p is required for the DNA damage and replication checkpoint and arrests the cell cycle at the G1/S transition, in S phase, or at the metaphase-anaphase transition in response to stresses (reviewed above). Nevertheless, Rad53p is not required for the meiotic pachytene checkpoint. Instead, a meiotic-specific version, Mek1p, is required for detecting DNA DSBs that arise as recombination occurs. In *S. pombe*, the Chk2 family member, Cds1, is mainly required for the S-phase DNA damage/replication checkpoint. Activated Cds1 arrests cells in S phase in response to unreplicated DNA or damaged DNA sensed during S phase. Whether Cds1 is required for the meiotic checkpoint is not yet known (reviewed above). Mammalian Chk2 is indispensable for G1/S, S, and G2/M checkpoint controls, but its role in the meiotic checkpoint is not clear. These functional and temporal divergences between the different CHK2 orthologs indicate that this protein kinase family displays an amazing degree of evolutionary plasticity (MEIER and AHMED 2001). This plasticity is further supported when one compares *C. elegans* and Drosophila *chk2*. While the former was shown to be required for meiotic chromosome pairing but dispensable for typical DNA damage/replication checkpoint responses induced by γ-irradiation or by HU, our results showed that *chk2* has no essential function in Drosophila meiosis. It is involved, however, in monitoring DSBs induced by γ-rays, which places it closer to its vertebrate homologs and makes it an excellent invertebrate model for studying human *chk2* function.

While this article was being reviewed, a study by ABDU *et al.* (2002) also found no evidence that *grp* transduces the DSB-activated meiotic checkpoint. However, because it is not completely clear that the *grp* allele is a null mutant, the results cannot rule out the possibility that *grp* acts as a transducer. ABDU *et al.* (2002) also present evidence that *chk2* transduces the DSB signal. This is surprising, given that our own results with a *chk2* null mutant fail to show suppression, suggesting that *chk2* is not the transducer. The most likely explanation for this discrepancy in the results is a difference in the background of the strains used. Resolving this contradiction requires a number of follow-up experiments.

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TABLE 2

Dorso-ventral patterning of the egg shell in *orb* mutants and *chk2; orb* double mutants

Maternal genotype	Wild-type dorsal appendages (%)	Fused dorsal appendages (%)	No dorsal appendages (%)	Hatching rate (%)
Control *Df(2L)be408,P[w+, CG10728+]* or *Df(2L)pr2b,P[w+,barren+]/CyO; $orb^{mel}/orbF^{343}$*	0/89 (0)	3/89 (3.4)	86/89 (96.6)	0/89
Df(2L)be408,P[w+, CG10728+]/ Df(2L)pr2b, P[w+, barren+]; $orb^{mel}/orbF^{343}$	0/175 (0)	12/175 (6.8)	163/175 (93.2)	0/175

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