Caenorhabditis elegans msh-5 Is Required for Both Normal and Radiation-Induced Meiotic Crossing Over but Not for Completion of Meiosis

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ABSTRACT

Crossing over and chiasma formation during *Caenorhabditis elegans* meiosis require *msh-5*, which encodes a conserved germline-specific MutS family member. *msh-5* mutant oocytes lack chiasmata between homologous chromosomes, and crossover frequencies are severely reduced in both oocyte and spermatocyte meiosis. Artificially induced DNA breaks do not bypass the requirement for *msh-5*, suggesting that *msh-5* functions after the initiation step of meiotic recombination. *msh-5* mutants are apparently competent to repair breaks induced during meiosis, but accomplish repair in a way that does not lead to crossovers between homologs. These results combine with data from budding yeast to establish a conserved role for Msh5 proteins in promoting the crossover outcome of meiotic recombination events. Apart from the crossover deficit, progression through meiotic prophase is largely unperturbed in *msh-5* mutants. Homologous chromosomes are fully aligned at the pachytene stage, and germ cells survive to complete meiosis and gametogenesis with high efficiency. Our demonstration that artificially induced breaks generate crossovers and chiasmata using the normal meiotic recombination machinery suggests (1) that association of breaks with a preinitiation complex is not a prerequisite for entering the meiotic recombination pathway and (2) that the decision for a subset of recombination events to become crossovers is made after the initiation step.

GENETIC recombination during meiosis is distinguished from mitotic recombination in several fundamental respects (Petes *et al.* 1991; Paques and Haber 1999). First, the frequency of detectable recombination events between homologous chromosomes is elevated by several orders of magnitude over mitotic recombination frequencies. Second, meiotic recombination events more frequently involve crossing over, or reciprocal exchange, between the participating chromatids. These crossover recombination events are crucial for generating chiasmata, physical connections between homologous chromosomes that persist until the metaphase/anaphase transition and allow the homologs to orient toward opposite poles of the meiosis I spindle (Jones 1987; Hawley 1988).

Crossover recombination during meiosis proceeds by a specialized double-strand break (DSB) repair pathway that has been modified from the vegetative/mitotic pathway by several conserved meiosis-specific or meiosis-enriched components (Roeder 1997; Paques and Haber 1999). Meiotic recombination events are apparently initiated by the deliberate enzymatic induction of DSBs. Direct evidence for meiosis-induced DSBs that serve as the recombination-initiating events comes from budding yeast (Sun *et al.* 1989; Cao *et al.* 1990), where such breaks are generated by the activity of Spo11p, a meiosis-enriched protein related to archaebacterial type II topoisomerases (Klapholz *et al.* 1985; Bergerat *et al.* 1997; Keeney *et al.* 1997). This mechanism for initiation of meiotic recombination is conserved across kingdoms; Dernburg *et al.* (1998) showed that meiotic recombination in *Caenorhabditis elegans* not only requires the nematode *SPO11* ortholog (*spo-11*), but also that artifically induced DNA breaks could bypass the requirement for *spo-11*. Spo11 homologs are likewise required for meiotic recombination in fission yeast and Drosophila, and for formation of meiosis-induced DSBs recently identified in fission yeast (Lin and Smith 1994; McKim *et al.* 1998; McKim and Hayashi-Hagihara 1998; Cervantes *et al.* 2000).

Another meiosis-enriched protein family with a widely conserved role in meiotic recombination includes Msh4p from budding yeast (Ross-Macdonald and Roeder 1994) and its ortholog HIM-14 from *C. elegans* (Zalevsky *et al.* 1999). Msh4p and HIM-14 are members of the MutS protein family, but unlike MutS and several other family members, they do not function in mismatch repair but rather have become specialized to function in meiotic recombination. Both HIM-14 and Msh4p act to promote crossover formation; crossovers are eliminated in *him-14* mutant worms and reduced by 50–70%

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in yeast *msh4* mutants. Moreover, Msh4p appears to function specifically in promoting the crossover outcome of meiotic recombination events, since interhomolog gene conversion frequencies are unaffected in the yeast *msh4* mutant.

Because crossovers are required to direct homolog segregation at meiosis I, the decision for a subset of recombination events to become crossovers is crucial for the genetic viability of the organism. How and when the crossover decision is made, and how this decision is subsequently enforced, are not known. In principle, the initial decision might be made at any of several steps. It could be made at or prior to the time of enzymatic initiation of recombination, perhaps by assembly of a preinitiation recombination complex that is predisposed to perform crossover recombination. Alternatively, the decision might be made at the strand invasion step; it has been proposed, for example, that the geometry of strand invasion could determine whether resolution of the resulting intermediates leads to a crossover or noncrossover product (Storlazzi *et al.* 1996). Further, the decision could be made after strand invasion by formation or stabilization of an intermediate that is specifically required to allow the crossover outcome or by imposing constraints on the eventual mode of resolution of a DNA intermediate that is common to both crossover and noncrossover pathways. Msh4/HIM-14 might conceivably act at any of these steps, or it might function at the time of resolution to enforce a bias imposed at an earlier time (Ross-Macdonald and Roeder 1994; Paques and Haber 1999; Zalevsky *et al.* 1999).

Experiments showing that artificially induced breaks can bypass the requirement for the recombination-initiating enzyme SPO-11 for both crossing over (Thorne and Byers 1993; Dernburg *et al.* 1998) and chiasma formation (Dernburg *et al.* 1998) suggest that the crossover decision is most likely made downstream of the initiation event. This interpretation depends, however, on the unproven assumption that the artificially induced events are in fact being generated by the "normal" crossover recombination pathway, an assumption we test in the current work.

We report here our analysis of a gene encoding another component of the crossover recombination machinery, *C. elegans msh-5*. While meiosis- or germline-enriched orthologs of this MutS family member have been identified in yeast, mice, and humans (Hollingsworth *et al.* 1995; Chu *et al.* 1998; Her and Doggett 1998; Winand *et al.* 1998; Bocker *et al.* 1999; de Vries *et al.* 1999; Her *et al.* 1999), efforts to define a conserved biological function for Msh5 proteins have been confounded by marked differences between the phenotypes observed in yeast and mice when *MSH5/Msh5* is eliminated. Loss of *MSH5* function in yeast leads to a specific deficit in crossover recombination events, but cells progress through meiosis and sporulate efficiently (Hollingsworth *et al.* 1995; N. Hollingsworth, personal communication). In contrast, germ cells in the mouse *Msh5−/−* mutant rarely progress beyond the zygotene stage of meiotic prophase and exhibit impaired and aberrant synapsis followed by massive apoptotic cell death (de Vries *et al.* 1999; Edelmann *et al.* 1999). Our investigation of *C. elegans msh-5* allows us to establish a conserved role for Msh5 proteins specifically in promoting the crossover outcome of meiotic recombination events. Further we show that most crossovers and chiasmata generated by artifically induced DNA breaks occur via the normal meiotic recombination pathway that requires *msh-5*. We discuss the implications of these findings for the mechanism of meiotic recombination, particularly regarding requirements for commitment to crossover recombination.

MATERIALS AND METHODS

Strains and maintenance: General methods for culturing *C. elegans* strains were as described in Brenner (1974), Wood (1988), and Epstein and Shakes (1995). Except where noted, all experiments were performed at 20°. The wild-type strain background was Bristol N2, and the following mutations and chromosome rearrangements were used (Riddle *et al.* 1997; Dernburg *et al.* 1998; this work):

LGIV: dpy-20(e1282ts), spo-11(ok79), unc-30(e191), msh-5(me23, me45), dpy-4(e1166), nT1[unc(n754dm) let]
LGX: dpy-3(e27) unc-3(e151).

The Bergerac strain RW7000 and STS markers therein were used for initial mapping of the *me23* mutation (Williams *et al.* 1992). Some strains were kindly provided by the Caenorhabditis Genetics Center.

"Green eggs and Him" screen: Worms homozygous for the integrated transgene array *yIs34* [a gift from M. Nicoll and B. Meyer, containing a *Pxol-1::gfp* transcriptional reporter (Nicoll *et al.* 1997) together with the *rol-6* (pRF4) transformation marker (Mello *et al.* 1991)] were mutagenized with ethyl methanesulfonate following standard procedures and allowed to produce F_1 progeny for ~12 hr (Figure 1). F_1 hermaphrodites were grown to adulthood, and when they had been gravid for 12–24 hr, their embryos were harvested by hypochlorite treatment and allowed to hatch in the absence of food to synchronize them as L1 larvae (Epstein and Shakes 1995). These F_2 larvae, most of which were hermaphrodites, were then allowed to resume growth in liquid culture to minimize the possibility of mating with spontaneous males in the population. After they became gravid, worms were pelleted, washed, and resuspended in M9 in the absence of food for 4 hr; this treatment causes them to cease egg laying, allowing the embryos *in utero* to age. This procedure maximizes the number of embryos present in the uterus that are in the gastrulation stage, when *Pxol-1::gfp* is expressed. Batches of worms were transferred to plates in 100 μl 10 mm NaAzide in M9, and plates were screened using epifluorescence microscopy for the presence of worms containing embryos exhibiting green fluorescent protein (GFP) fluorescence. Since the reporter expresses GFP only in XO (male) embryos, most hermaphrodites will contain no green eggs, whereas worms that produce a high frequency of XO embryos will contain green-fluorescing eggs. Candidate mutants were plated individually, allowed to produce self-progeny, and then were crossed with wild-type (Bristol N2) males to produce cross-progeny.

Genetic mapping: The *me23* mutation was mapped to chromosome IV as in Williams *et al.* (1992). *me23* was then localized to within ~0.1 cM of *unc-30* by multi-point crosses, as summarized below; for each heterozygote shown, the number of the total recombinant progeny selected that had a crossover in a given interval is shown in parentheses between the markers flanking that interval:

dpy-20 unc-30/me23 *dpy-20* (25/25) (*unc-30 me23*)
unc-30 dpy-4/me23 (*unc-30 me23*) (33/33) *dpy-4*

Experiments using *dpy-20* were performed at 23°.

cDNA and Northern analyses: cDNAs corresponding to the *msh-5* gene were generated by RT-PCR as follows: To generate a cDNA corresponding to the 3′ end of the gene, first-strand cDNA synthesis was primed with GACTCGAGTCGACATC $GA(T)_{17}V$ (where variant *V* is A = G = C), and the cDNA was amplified using a gene-specific primer, CGTACCGAATCAAG TTAGTAGTGG, and a 3′ end adapter primer, GACTCGAGT CGACATCGA; the cDNA obtained was 2.4 kb in length. To generate a cDNA from the 5′ end of the gene, first-strand cDNA synthesis was primed using a gene-specific primer, CAGACGGAAGTGTTGGTCG, and the cDNA was amplified using a nested primer, CGCATGTCTAGCTGGCACGAAA CTTCC, in conjunction with ATAAGAATGCGGCCGCGGTT CAAATGTCCACTCGATGG (which includes the initiation codon plus and added *Not*I site); the cDNA obtained was 2 kb in length. A 2.6-kb partial *msh-5* cDNA clone, yk353a3, was obtained from Dr. Yuji Kohara of the National Institute of Genetics, Mishima, Japan. A composite cDNA (4.2 kb in length) was constructed using the 5′ RT-PCR product and yk353a3, and this clone as well as the RT-PCR products were sequenced. The entire coding sequence has been deposited in GenBank (accession no. AF271389). A previously reported partial cDNA generated by 5′ rapid amplification of cDNA ends indicated that the *msh-5* message is *trans*-spliced to SL1 five nucleotides upstream of the predicted initiation codon (Winand *et al.* 1998).

A Northern blot containing RNA from wild-type adults and *glp-4(bn2)* adults lacking a germline was hybridized with the 2.4-kb RT-PCR product from the 5′ end of the gene (labeled with [^{32}P]dATP by random priming, Sambrook *et al.* 1989). Following decay of the hybridization signal, the blot was reprobed with the 2-kb RT-PCR product corresponding to the extreme 3′ end of the gene. Both probes detected a single germline-dependent 4.4-kb transcript. Previous probing of the same blot demonstrated equal loading of RNA samples (Dernburg *et al.* 1998).

Detection of achiasmate chromosomes in oocyte nuclei: To assess frequencies of achiasmate oocyte chromosomes, worms were fixed with Carnoy's fixative and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described in Villeneuve (1994). Since individual univalents or bivalents in some nuclei may lie too close to each other to be resolved unambiguously, this method underestimates the frequency of achiasmate chromosomes.

Imaging of meiotic chromosome morphology: To evaluate the morphology of well-preserved pachytene nuclei at high resolution, worms were dissected in modified egg buffer (27.5 mM HEPES pH 7.4, 130 mM NaCl, 53 mM KCl, 2.2 mM $MgCl_2$, 2.2 mM $CaCl_2$) containing 15 mM NaAzide and 0.1% Tween-20, and then fixed by addition of an equal volume of 0.8% ethylene glycol-bis(succinimidylsuccinate) (Pierce, Rockford, IL) in egg buffer containing 20% DMSO. The tissue was sandwiched between a positively charged glass slide (SuperFrost Plus, Fisher) and a siliconized coverslip and incubated for 30 min at room temperature. The slide was then frozen by immersion in liquid nitrogen, the coverslip was quickly removed, and the sample was transferred to 95% ethanol at −20°. The slides were then postfixed in 2× SSC (0.3 M NaCl, 0.03 M Na citrate) containing 5% formaldehyde at room temperature for 30 min, washed with several changes of 2× SSC containing 0.1% Tween-20 (2× SSCT), and stained with 0.5 μg/ml DAPI in the same buffer. Samples were mounted for microscopy in 90% glycerol containing 3.6% N-propylgallate, buffered to pH 8 with Tris base. Imaging was performed using a DeltaVision wide-field optical sectioning microscope; three-dimensional data stacks were collected at 0.2-μm Z-spacing using a 100×, 1.35N.A. Nikon objective lens. Deconvolution was performed using an empirically measured point-spread function. Projections were generated using a maximum-intensity algorithm.

***In situ* hybridization:** To assess homolog pairing, fluorescence *in situ* hybridization was performed using DNA probes to various loci. These probes were generated from pools of 3–6 cosmids or by PCR amplification of the 1-kb repeated unit of the 5S rDNA locus. All probes were enzymatically digested and 3′ end-labeled with fluorescent nucleotides using terminal transferase as described in Dernburg (1999). Hybridization conditions were as described in detail in Dernburg (1999). Briefly, samples fixed as described above were equilibrated in 2× SSCT containing 50% formamide. Probes were then added in hybridization solution (50% formamide, 3× SSC, 10% Dextran sulfate) and overlaid with a glass coverslip. The tissue and probes were simultaneously denatured by heating to 91° for 2 min in a humidified chamber and then allowed to anneal overnight at 37°. Samples were washed at 37° in 2× SSCT containing 50% formamide for 1 hr, reequilibrated in 2× SSCT, and then stained with DAPI and imaged as above.

Genetic recombination frequencies: Males of genotype *msh-5(me23)/+* were crossed with hermaphrodites of genotype *msh-5(me23)/+; dpy-3 unc-3.* Cross-progeny hermaphrodites were picked to single plates and transferred daily for 2 days. Complete broods were then examined for Unc Dpy, wild-type, Unc non-Dpy, and Dpy non-Unc recombinant progeny. *msh-5/msh-5* animals were distinguished from control (*msh-5/+* or *+/+*) animals based on production of inviable zygotes. Recombination frequency (*p*) for the control was calculated as $p = 1 - (1 - 2R)^{1/2}$, where *R* is the frequency of phenotypic recombinant progeny (Brenner 1974). The recombination frequency calculated for *msh-5* is a weighted average of the recombination frequency calculated from scoring hermaphrodite (XX) progeny and that calculated from scoring male (XO) progeny (where $p = R$): $[p_{\text{hermaphrodite}}$ (2 × [no. of hermaphrodites]) + p_{male} (no. of males)]/total no. of X chromosomes sampled. Map distances in cM = 100 × *p*. The percentage of recombinant X chromosomes derived from irradiated *msh-5* or *spo-11* mutants was calculated in the same way; raw data for *spo-11* were taken from Dernburg *et al.* (1998).

Apoptosis assays: Acridine orange (AO) staining of apoptotic germ cells in *msh-5(me23)* and wild-type hermaphrodites was carried out using modifications of a procedure developed by Gumienny *et al.* (1999). Late-stage L4 hermaphrodites were picked and aged for 20 hr. Adult worms were picked into 100 μl 25 μg/ml AO in M9 in brown 1.5-ml tubes and nutated for 2 hr at room temperature. Care was taken to transfer some bacteria (for food) along with the worms so that the worms would continue feeding during the staining period, facilitating uptake of the dye. Worms were allowed to recover for 10 min on bacterial lawns and then mounted in 60 μg/ml levamisole in M9 onto agar pads on microscope slides and examined by epifluorescence microscopy to assess the number of AO-positive nuclei per gonad arm. In some animals, both gonad arms could be scored; in others, the position of the autofluorescent intestine obscured one of the two gonad arms.

For *msh-5(me45)* (and concurrently scored wild-type controls), worms were stained 24 hr after they were picked as late-stage L4s.

Irradiation experiments: Late-stage L4 worms were exposed to 5000 rad of γ rays from a ^{137}Cs source and were subsequently assayed for progeny viability, chiasma formation, and crossing over as described above and in RESULTS. In addition to the time points shown in Table 3, we also scanned oocyte nuclei at 8, 10, 12, and 14 hr after irradiation of *msh-5(me23)* mutant germlines; we did not find any time point with a noticeable increase in the frequency of bivalents.

We estimated the minimum number of DNA breaks (capable of serving as initiating lesions for meiotic recombination) that are being generated by this procedure as follows: We pooled the data for γ-ray-induced chiasma formation for all three time points for *spo-11* to calculate that 91% of homolog pairs had a chiasma. Reasoning that chiasmate homolog pairs must have suffered at least one break, we took the fraction of homolog pairs that lacked a chiasma, 0.09, as an upper estimate of the fraction of homolog pairs that lacked a break, $f(0)$. We then calculated the mean number of breaks per homolog pair (m) using the Poisson equation $f(0) = e^{-m}$. The resulting lower limit estimate of the mean number of breaks per chromosome is $m/2$, or 1.2. This is likely an underestimate for several reasons. First, we do not know whether a single DSB per homolog pair will necessarily yield a crossover event. Second, the chromosomes range in size from 14 to 20 Mb, so if initiation-competent lesions are Poisson-distributed with respect to DNA length, shorter chromosomes will be more likely to lack a break than longer chromosomes, and longer chromosomes will have more breaks.

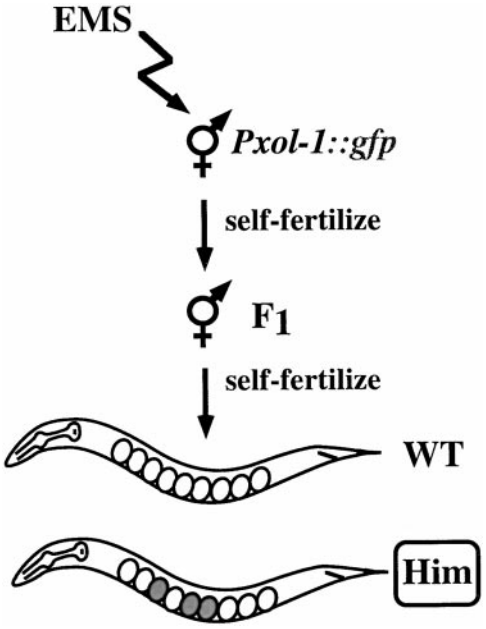


FIGURE 1.—"Green eggs and Him" screen for mutants with high meiotic nondisjunction. Hermaphrodites (XX) carrying a *Pxol-1:gfp* reporter transgene, which expresses GFP only in male (XO) embryos, were mutagenized and allowed to produce F_2 descendants. Most F_2 hermaphrodites produce only hermaphrodite progeny and thus contain no green embryos. F_2 hermaphrodites with defects in meiotic chromosome segregation exhibit a "high incidence of males" (Him) phenotype and can be identified by the presence of green-fluorescing XO embryos in their uteri. See MATERIALS AND METHODS for details.

RESULTS

Isolation of *C. elegans msh-5* mutants: To identify gene products required for normal regulated crossing over during meiosis, we used a variation of our earlier screen for *C. elegans* mutants defective in meiotic chromosome segregation (VILLENEUVE 1994). Previously, we identified meiotic mutants on the basis of a "Him" (high incidence of males) phenotype: Whereas self-fertilizing hermaphrodites (XX) normally give rise to male (XO) progeny at a frequency of 0.2%, mutants that missegregate chromosomes in meiosis can produce up to 35–45% XO male self-progeny (HODGKIN *et al.* 1979; HERMAN *et al.* 1982; VILLENEUVE 1994; DERNBURG *et al.* 1998; ZALEVSKY *et al.* 1999; ZETKA *et al.* 1999). While this screen was successful in identifying several key components required either for recombination *per se* (ZALEVSKY *et al.* 1999) or for homolog pairing and/or synapsis (VILLENEUVE 1994; A. MACQUEEN and A. VILLENEUVE, unpublished results), it has two drawbacks. First, the screening procedure involves progeny testing of individually plated worms, limiting the number of genomes that can be screened practically. Second, since most meiotic mutants missegregate all chromosomes, they produce broods consisting mostly of inviable aneuploid embryos, with only a few adult survivors (HODGKIN *et al.* 1979; VILLENEUVE 1994; DERNBURG *et al.* 1998; ZALEVSKY *et al.* 1999; ZETKA *et al.* 1999); some individual meiotic mutants may produce no surviving progeny and thus would appear indistinguishable from maternal-effect lethal mutants in this screen.

Our current screen circumvents both of these problems by making use of a *Pxol-1::gfp* reporter that expresses GFP specifically in XO embryos (NICOLL *et al.* 1997; Figure 1). Hermaphrodites are screened *en masse* to identify mutants that have green-fluorescent embryos *in utero*. Further, inviable aneuploid embryos can express the reporter, allowing meiotic mutants to be identified even if they produce no surviving progeny. Such mutants can frequently be recovered by mating them with wild-type males to provide euploid sperm, thereby increasing the probability of obtaining viable euploid zygotes. Among the meiotic mutants isolated in this ongoing "Green eggs and Him" (inspired by GEISEL 1960) screen, we identified two (*me23* and *me45*) containing mutations in predicted gene F09E8.3 (*C. ELEGANS* SEQUENCING CONSORTIUM 1998), which encodes the *C. elegans* ortholog of the meiosis-specific MutS family member Msh5 (EISEN 1998).

Genetic mapping localized the *me23* mutation to within 0.1 cM of the *unc-30* gene (see MATERIALS AND METHODS), which is 30 kb from F09E8.3, now renamed *msh-5*. Further, functional knockout of the *msh-5* gene using transgene-mediated cosuppression induced a robust *me23* phenocopy (DERNBURG *et al.* 2000). Sequencing of this candidate gene revealed that the *me23* allele contains a G → A transition that destroys the invariant

G in the splice-acceptor site of the third intron of the *msh-5* gene (Figure 2A). RT-PCR experiments indicate that the *me23* mutant fails to accumulate spliced *msh-5* message, corroborating the expected splicing defect. Further, sequencing of larger PCR products obtained following RT-PCR using RNA from the *me23* mutant showed that introns downstream of the defective splicing signal were retained, suggesting that only unprocessed transcripts were available as template. Thus the *me23* mutation is expected to severely reduce or eliminate *msh-5* function.

The second *msh-5* allele, *me45*, was identified after most of the analysis reported in this article was complete. *me45* is expected to be a null allele, since it contains a G → A transition that results in a premature stop codon that truncates translation of the protein after amino acid 350, far upstream of the conserved ATP-binding domain and helix-turn-helix motif that define the MutS family and are required for activity of MutS family members both *in vitro* and *in vivo* (Haber *et al.* 1988; Haber and Walker 1991; Alani 1996; Alani *et al.* 1997; Pochart *et al.* 1997). The *me23* and *me45* alleles confer apparently equivalent mutant phenotypes; quantitative phenotypic analysis was performed using *msh-5(me23)*, and several key findings were confirmed for *msh-5(me45)*.

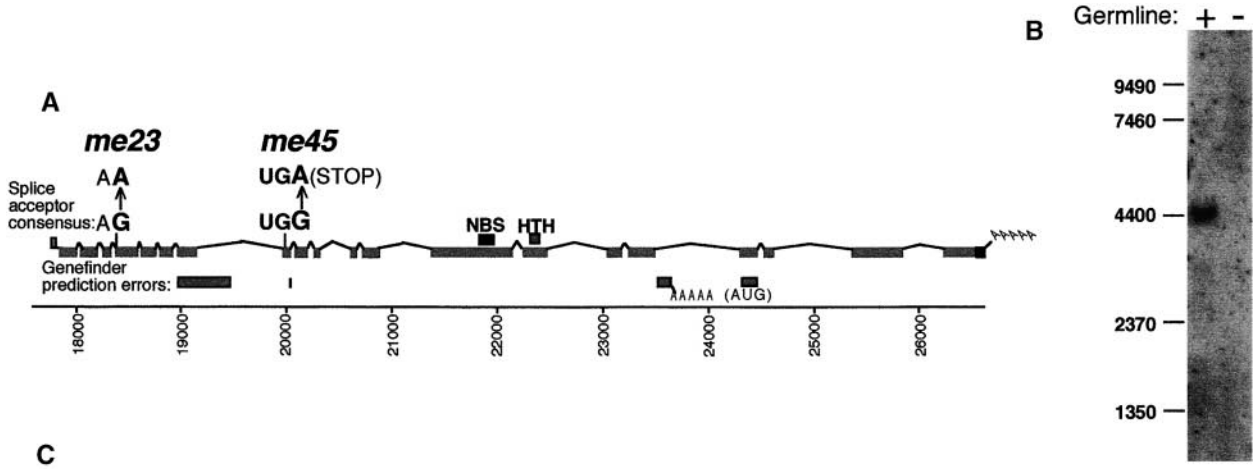
Figure 2A shows the modified *msh-5* gene structure deduced from sequencing of cDNAs obtained by RT-PCR and from the Kohara expressed sequence tag library. While most of the intron/exon boundaries in the gene structure predicted by Genefinder and displayed in ACeDB (Durbin and Thierry-Mieg 1991) were confirmed, several differences were identified. First, the splice donors used at the 3′ ends of exons 7 and 8 differed from those predicted, as noted in a previously reported sequence of a partial cDNA (Winand *et al.* 1998). Second, the 17th exon predicted by Genefinder (which is preceded by a nonconsensus splice acceptor site) is *not* used; instead the 16th exon is spliced to a 5′ acceptor site just upstream of the "AUG" of a falsely predicted gene, F09E8.4, and the remaining splice sites predicted for F09E8.4 were verified. The assembled composite *msh-5* cDNA (including coding region and untranslated region, but excluding poly(A) tail) is 4.2 kb in length. This corresponds well with the size of the single transcript identified by Northern blot (Figure 2B). The 4.4-kb *msh-5* transcript is present in wild-type adult worms but absent in worms lacking a germline, consistent with *msh-5* having a meiosis-specific function. The open reading frame encodes a predicted protein of 1369 amino acids; the 906-amino-acid N-terminal portion shares similarity with the yeast, mouse, and human orthologs along their entire lengths (Figure 2C). The predicted *C. elegans* MSH-5 protein also has a 463-amino-acid C-terminal tail not present in the yeast, mouse, or human proteins; the functional significance of this tail, if any, is unknown.

***C. elegans msh-5* is required for meiotic crossing over and chiasma formation:** Both *msh-5(me23)* and *msh-5(me45)* confer a collection of phenotypes diagnostic of a defect in meiotic reciprocal recombination; quantitation is reported for *me23*. Homozygous *msh-5* mutant hermaphrodites are morphologically normal, lay eggs at a wild-type rate, and produce normal numbers of embryos. Of these embryos, 97.9% fail to hatch (n = 1326), however, and among the progeny that survive to adulthood, 42% are male (n = 369 adult survivors), indicating a severe defect in meiotic chromosome segregation. This *msh-5* mutant phenotype is essentially identical to that produced by a null mutation in *him-14*, which encodes the *C. elegans* ortholog of Msh4 (Zalevsky *et al.* 1999), the heterodimer partner of Msh5 in yeast and humans (Pochart *et al.* 1997; Winand *et al.* 1998; Bocker *et al.* 1999).

As with the *him-14* mutants, cytological analysis of DAPI-stained chromosomes late in meiotic prophase demonstrated an absence of chiasmata in *msh-5* mutant oocytes. At this stage (termed diakinesis), homologous chromosomes have lost the side-by-side association characteristic of earlier stages of meiotic prophase, but in wild-type oocytes they remain attached by chiasmata that formed as a consequence of crossover recombination that occurred at a previous stage (Jones 1987). Whereas 6 DAPI-stained bodies are detected in wild-type oocytes (corresponding to six bivalents, *i.e.*, homolog pairs attached by chiasmata), 12 separate DAPI-stained bodies (univalents) were clearly resolved in the majority of *msh-5* mutant oocytes (Figure 3). Specifically, an average of 11.7 DAPI-stained bodies were resolved in 141 oocyte nuclei from 33 *msh-5(me23)* hermaphrodites, indicating that nearly all, if not all, homolog pairs lacked chiasmata. The absence of chiasmata can readily account for the observed defect in meiotic chromosome segregation.

Measurement of genetic recombination frequencies indicates that the absence of chiasmata in *msh-5* mutants results from failure to form crossovers rather than premature release of chiasmata (Table 1). For a 39-cM interval corresponding to 80% of the X chromosome, the crossover frequency measured in the *msh-5(me23)* mutant was reduced to 1% of the wild-type frequency: Only one recombinant was observed among 248 progeny scored. Since this assay detects recombination in both male and female germ cells, this extreme reduction in recombination frequency indicates that crossover recombination is severely defective in both spermatocyte meiosis and oocyte meiosis in *msh-5* mutant hermaphrodites.

Intimate pairing and alignment of homologs are normal in *msh-5* mutants: We evaluated whether *msh-5* mutations might reduce crossing over by perturbing the organization of meiotic chromosomes during prophase. Specifically, we wished to establish whether intimate association between homologous chromosomes de-



C

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C.e.      1 MSTRWRYYNSKRGNGFRGRGRGRGRGTSLTAVALPRDDNFHKGAQDGAYFKD-MPMDPEQFRDETVLSLSFAQGMLGAAY
M.m.      1 MAFRATPGRTPPGPGPR-----SG----IPSASFPSPQ--PPMAGPGGIEEE-DEEEP----AEIHLCVLWSSGYLGIAY
S.c.      1 MSHEWLISASETMRSIG-----NGEGLRDKGAVVANNDG-EFNEGDTNREEDSSTIFSFDFDEEEIVMCIDFSGGKLGCSI

C.e.     80 YEQSSQLLKIMN-------------DISEDLEFR------FLKRLIDDVKPTLIIANRSQDLEFIKFLTTRYIDPQEKIYE
M.m.     65 YDTSDSTIHFMP-------------DAPDHESLK------LLQRVLDEINPQSVVTSAKQDEAMTRFLGKLAS-----ES
S.c.     75 LDYHTKTLKAFDQDYVVNKTTISSHDLIDDADMSSNDISLLLGLLIMEANPTVCLVPARLEDWIFDYIKTKCD------S

C.e.    141 DGTTEEGTSEDTVPTWDSSLAYSTDETTAEKEEKEEDEDDDDEGLPAKLNKLPNNFFRMSRAIERLKAMAGSHDSSMTEE
M.m.    121 HR-EPKGPELILLPSVDFGPEIS-------------------------KQRLLSGNYSFIS-------------DSMTAT
S.c.    149 INCRLELQPIKRFKKWDLLQSLQ-------------------------LRGHDNQTILN---------------DILSN

C.e.    221 DKYIIIKMRFDIEAVNMIRSFGALLLFLDETRMGVTDDPLSVTSP--IKSIKTFTLGNLVEIDFNTIQALDILPKETEN-
M.m.    162 EKILFLSSIIPFDCVLTVRALGGLLKFLSRRRIGVELEDIDVGVP--ILGFKKFVLTHLVSIDQDTYSVLQIFKSESHPS
S.c.    188 SKFTTTVTLGTVGCILLANHEQLGEYNDSTASSNMVTGRLVQNAFEDVIHGIRYIDIRDRMVLDENTISALHIFPTAHKLG

C.e.    298 ----KKTFGQGRSLYQLMDKCRSTVGKKCLRKWFRNPTTDRDDLVSRQKCVHYFKQDWNAEVTAKLSSILGRVKALNSVF
M.m.    240 VYKVASGLKEGLSLFGILNRCRCKWGQKLLRLWFTRPTRELRELNSRLDVIQFFLMPQNLDMAQMLHRLLSHIKNVPLIL
S.c.    268 --HDKMMRNGFFSVFELFNQVSSDYARRILKSWLINPLTNKKRIETRYSILRTLLDKQNAIIFSDLSQSIKRCPDAFGFI

C.e.    374 QKFQSGTAQLIHWECFVSTVNALVEILNIIRQTPISKEFP--VESDLLREVSEIAV-----IAGSILNFAESKIQGRVTV
M.m.    320 KRMKLSHTKVSDWQVLYKTVYSALGLRDACRSLPQSIQLFQDHAQEFSDDLHHIAS-----LIGKVVDFEESLAENRFTV
S.c.    346 NQLRSGKSTLGTWSKVASFLEKGIAIFQLVSSLKLGSDEAN-ILHDIKNKVDISALKECLRKVETVIDFDTSRDTKTLTI

C.e.    447 MNGIDEELDEIRDTYENMPMVLTAIAKQEEARLGLPPYSN-----------VACVYIPLVGFVLSVPRDYGVESQPD---
M.m.    395 LPNIDPDIDAKKRRLIGLPSFLTEVAQKELENLDSRIPS-----------CSVIYIPLIGFLLSIPRLPFMVEASDFEI
S.c.    425 NTGVDNRLDECRNIYNHLEGILLDVARETQIFLLNTMPQEDCKTTKSLEKLVNAVYIPQLGYLVTISVLMEPLLDGIPN-

C.e.    513 --MTLLYSTHEDLRVRNATTSRLDDEFGDILMRLIDSQTAILTLKTRVMKKKRSIIKLLSIASRIDVLISFGLIAAQNG
M.m.    463 EGLDFMFLSEDKLHYRSARTKELDTLLGDLHCEIRDQETLLMYQLQCQVLARASVLTRVLDLASRLDVLLALASAARDYG
S.c.    504 LQWEEIFRSSENIYFKNGRVLELDETYGDIYGAISDFEIELFSLQEQILRRKTQLTAYNILLSELEILLSFAQVSAERN

C.e.    591 WNCPALVDEPVIEAVELY---HPISVLVVKKSFVPNQVSSGR-----------DGIKASIITGPNACGKSVYMKSIGIMV
M.m.    543 YSRPHYS--PCIHGVRIRNGRHPLMELCAR-TFVPNSTDCGG-----------DQGRVKVITGPNSSGKSIYLKQVGLIT
S.c.    584 YAEPQLVEDECILEIING--RHALYETFLD-NYIPNSTMIDGGLFSELSWCEQNKGRIIVVTGANASGKSVYLTQNGLIV

C.e.    657 FLSHIGSFVPARHAKIGIVDRIVTRMFTVDSVLDGMSTFAKDVEQVALALRKATGNSLVIIDEFGKGTMTEVGLSLLASV
M.m.    609 FMALVGSFVPAEEAEIGVIDAIFTRIHSCESISLGLSTFMIDLNQVAKAVNNATEHSLVLIDEFGKGTNSVDGLALLAAV
S.c.    661 YLAQIGCFVPAERARIGIADKILTRIRTQETVYKTQSSFLLDSQQMAKSLSLATEKSLILIDEYGKGTDILDGPSLFGSI

C.e.    737 MTYMMNRGADRCPHIFLSSHFHALPNYIPLETNI-----------ATFLTFTVLREAGGK-----IKYLFRMTPGLVDC
M.m.    689 LRHMLALGPS-CPHVFVATNFLSLVQLQLLPQGP-----------LVQVLTMETCEDGED-----LVFFYQLCQGVASA
S.c.    741 M-LNMSKSEK-CPRIIACTHFHELFNENVLTENIKGIKHYCTDILISQKYNLLETAHVGEDHESEGITFLFKVKEGISKQ

C.e.    800 SFALSVAKEEGIPPPVIGRACRIYKALKAGT-LLKEIKAEVSNDNEKQLVEDMDVVLADEDGFMAAVESFVKRKKTSFCE
M.m.    751 SHASHTAAQAGLPDPLIARGKEVSDLIRSGK-PIKATNELLRR-NQ----------MENCQALVDKFLK-----LD-
S.c.    819 SFGIYCARVCGLSRDIVERAEELSRMINRGDDVVQQCGNLTEKEMR----------EFQKNQEIVKKFLS--------

C.e.    879 SSMRNVSEEIEKERSEASTPASKSRSTITARSNSVLSSRSMASVDQLSVLDALLPKKKKKKVTGSSMESSMSPDPFQEED
M.m.    810 --LEDPTLDLDIFISQEVLPAAP----TIL-----------------------------------------------
S.c.    879 -------WDLDLETTTTSENLRLKLKNFLR-----------------------------------------------

C.e.    959 EGTEGEEDQISAPVSRPTLPSVQKYASEEEKQQSINSRHSFSTRTAIHIPTPIQMGEAGGVKRPRSTSTSSPGPSASKSV

C.e.   1039 RTEVFKKTPNVKESQVLETPKQLSISSFLEPKFPSSEKDVISRVSERYLQSDPFKTPISDRRSQQSSRHSTPKNRSMNQS

C.e.   1119 LIQSARDTPHETIRSSNEVNPEFFNIFNFPDDSILKSQDTYIDPNVTPRSSSRRELRPDVSHSQNSQFGEVFSELGTQFSI

C.e.   1199 FNSQQSFPGNSMGTTNPDCSIFDDFFANSQDGEKKIDSTKTSMPIVNSDNFIFKTPEPRSSEKQRSLLKNKGQASNSSIS

C.e.   1279 PSSLILGQLAFGDVDQTPRPRGDNPIEFQYDVVDDDDPIFEEKNCSAPVFEFLKSNDDEEDDEFLKSFLETEGSLHIDTS

C.e.   1359 ADETIDRSKRSZ
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TABLE 1

Severe reduction in crossover frequency in the *msh-5(me23)* mutant

Genotype of parent	No. of recombinants	No. of progeny scored	Map distance (cM)[a]
+/(msh-5 or +); dpy-3 unc-3/++	520	1658	38.4
msh-5/msh-5; dpy-3 unc-3/++	1 hermaphrodite[b] 0 male	139 hermaphrodite 109 male	0.5[b]

[a] Map distances were calculated as described in MATERIALS AND METHODS.

[b] The one apparent Dpy non-Unc recombinant obtained produced no eggs, so its genotype could not be verified; it is possible that it was actually not a recombinant, but rather an aneuploid.

pends on *msh-5* function. Each nematode germline represents a time course of nuclei arranged in a temporal-spatial gradient from premeiotic stages through meiotic prophase stages and the meiotic divisions; nuclei at different stages can be distinguished by their morphological appearance in DAPI-stained preparations (SCHEDL 1997; DERNBURG *et al.* 1998). Using *in situ* hybridization, the spatial relationship between homologous chromosomes can be examined in the context of three-dimensional nuclear architecture (DERNBURG *et al.* 1998).

We found that premeiotic nuclei, nuclei from the "transition zone" (where meiotic prophase begins and homolog pairing is established, DERNBURG *et al.* 1998), and nuclei in the pachytene region (where homologous chromosomes are fully aligned and synapsed along their entire lengths) all appeared indistinguishable from wild type in *msh-5* mutant germlines. Moreover, the relative proportions of nuclei at the various stages were normal. Further, fluorescence *in situ* hybridization (FISH) experiments demonstrated that homologous chromosomes enjoyed normal intimate associations in both *msh-5(me23)* and *msh-5(me45)* mutants. Figure 4 shows high-resolution images of DAPI-stained chromosomes in wild-type and *msh-5(me23)* pachytene stage nuclei hybridized with FISH probes from two different chromosomes. These projections through 3D data stacks spanning whole nuclei reveal the characteristic pachytene morphology of parallel DAPI-stained cables, corresponding to the side-by-side aligned and synapsed homologs. For each FISH probe, a single focus of hybridization or closely spaced doublet is observed in both wild-type and *msh-5* nuclei, indicating intimate pairing of homologous sequences.

Survival of *msh-5* mutant germ cells: The most obvious phenotype of mice mutant for *Msh5* is severe hypogonadism and sterility in both males and females, a consequence of massive germ cell apoptosis (DE VRIES *et al.* 1999; EDELMANN *et al.* 1999). *C. elegans msh-5* mutants clearly are able to complete meiosis and produce embryos, and germ cell nuclei at all cytologically distinguishable stages are present in normal proportions in their germlines, so it is clear that they do not experience germ cell apoptosis on a scale comparable to that observed in *Msh5−/−* mutant mice. However, a modest increase in germ cell apoptosis could be compatible with other aspects of the *msh-5* phenotype, so we examined the frequency of apoptotic germ cell nuclei in *msh-5* mutant worms.

Germ cell apoptosis occurs as part of the normal physiology of the *C. elegans* germline; it has been estimated that approximately half of wild-type female germ cells eventually undergo apoptosis when they reach the end of the pachytene stage (GUMIENNY *et al.* 1999). These cell deaths are thought to play a role in maintaining a homeostasis between the "nurse cell" function of germline nuclei (*i.e.*, producing transcripts for packaging into oocytes) and the rate of oocyte production. The physiological cell deaths do *not* serve as a means of culling defective germ cells, since *ced-3* and *ced-4* mutants (which lack apoptosis) do not show an increase in embryonic lethality (GUMIENNY *et al.* 1999), nor do they exhibit an obvious mutator phenotype. In addition to the physiological germ cell deaths, GARTNER *et al.* (2000) recently showed that apoptosis of female germ cells can also be triggered by activation of a DNA damage checkpoint; this checkpoint-induced apoptosis is likewise restricted to late pachytene nuclei.

Our analysis of germ cell apoptosis indicates that the majority of female germ cells normally destined to sur-

FIGURE 2.—*msh-5* gene structure, expression, and protein alignment. (A) Intron/exon structure of the *msh-5* gene, indicating the positions of the *me23* mutation in the splice acceptor of the third intron and the *me45* Stop mutation. NBS indicates the position of the Walker homology nucleotide binding sequence that defines the MutS family, and HTH indicates the position of the predicted helix-turn-helix motif. Below the gene structure are indicated errors in the Genefinder-predicted gene structure (discussed in the text). Numerical scale corresponds to sequence coordinates of cosmid F09E8. (B) Northern blot containing RNA from young adult wild-type worms and *glp-4(bn2ts)* worms that lack a germline, showing the single 4.4-kb germline-dependent *msh-5* transcript. Previous probing of the same blot demonstrated equal loading of RNA samples (DERNBURG *et al.* 1998). (C) Alignment of the *C. elegans* MSH-5 predicted protein sequence with the mouse Msh5 and yeast Msh5p sequences. The Walker homology ATP-binding motifs are boxed in black, and the predicted helix-turn-helix motif is boxed in white.

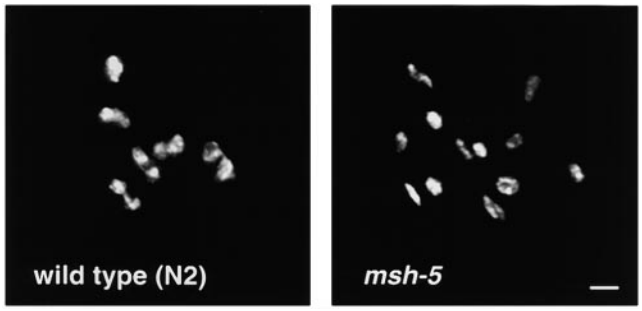

FIGURE 3.—Absence of chiasmata in the *msh-5* mutant. DAPI-stained oocyte nuclei at diakinesis, the last stage of meiotic prophase. Both sides show a projection of a 3D data stack encompassing an entire nucleus. Six bivalents are seen in the wild-type nucleus, corresponding to the six pairs of homologous chromosomes attached by chiasmata. Twelve univalent chromosomes are seen in the *msh-5(me23)* mutant nucleus, indicating an absence of chiasmata between homologs. Bar, 2 μm.

vive do indeed survive in *msh-5* mutant hermaphrodites. We detected an average of 7.7 ± 2.7 apoptotic nuclei per gonad arm by AO staining of *msh-5(me23)* germlines (n = 64), compared with an average of 4.7 ± 1.5 apoptotic nuclei per gonad arm in the germlines of age-matched wild-type hermaphrodites (n = 38) that were scored concurrently. We confirmed a high efficiency of germ cell survivorship in *msh-5(me45)* mutant hermaphrodites; we detected an average of 5 ± 2.2 apoptotic nuclei in *msh-5(me45)* germlines (n = 21) compared with 6 ± 1.9 in concurrently scored wild-type controls (n = 20). These results contrast sharply with a 13-fold increase in frequency of apoptotic nuclei elicited when the DNA damage checkpoint is triggered by depletion of the *C. elegans* Rad51 homolog (GARTNER *et al.* 2000). While the small difference between *msh-5(me23)* and wild type is statistically significant, it would nevertheless indicate only a very modest decrease in survivorship of *msh-5(me23)* mutant germ cells relative to wild type. It is estimated that in wild-type germlines, one female pachytene nucleus undergoes apoptosis for each nucleus that goes on to complete meiosis (GUMIENNY *et al.* 1999). Accordingly, a 1.6-fold increase in the frequency of apoptotic nuclei would translate to 1.6 nuclei undergoing apoptosis for every nucleus that completes meiosis, or an apoptosis rate of 62%; this would represent a 24% increase over the wild-type apoptosis rate of 50%, or a 24% decrease in germ cell survivorship.

Artifically induced DNA breaks do not bypass the requirement for *msh-5*: DERNBURG *et al.* (1998) showed previously that DNA breaks generated by γ-irradiation could bypass the requirement for the recombination-initiating enzyme SPO-11. This treatment very efficiently generated not only crossover recombination events, but also functional chiasmata capable of holding homologs together (as assayed cytologically) and an increase in progeny viability, presumably reflecting improved fidel-

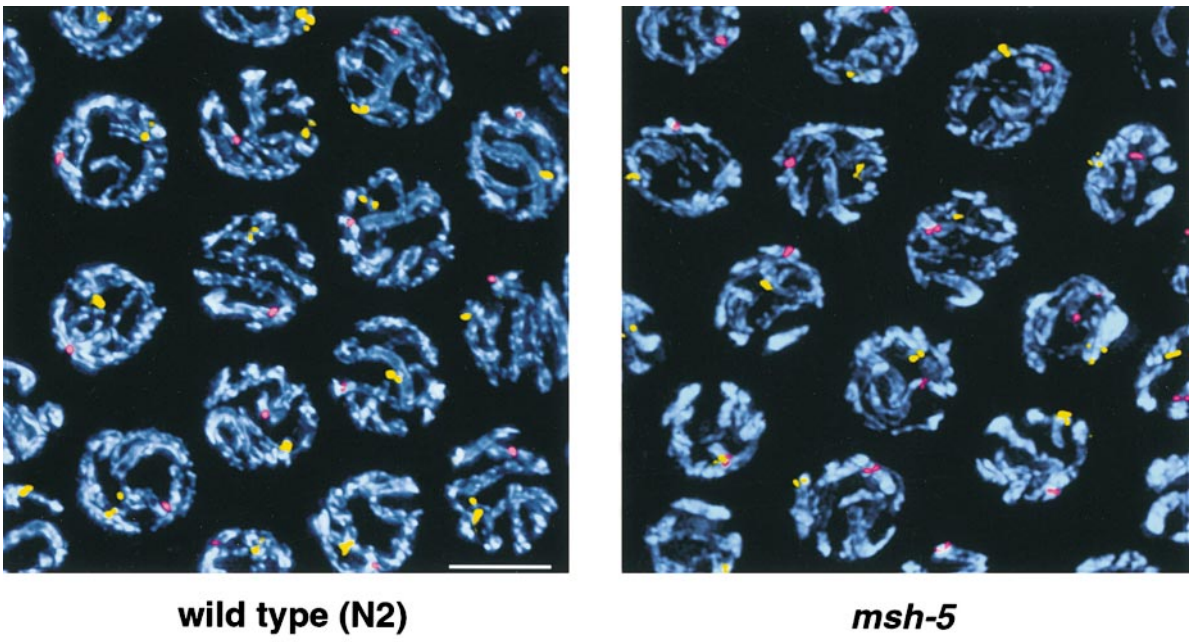

FIGURE 4.—Normal intimate pairing and alignment of homologous chromosomes in *msh-5* pachytene nuclei. Comparison of fields of DAPI-stained pachytene nuclei from wild type and the *msh-5(me23)* mutant reveals normal pachytene chromosome morphology. Homologous chromosomes are aligned along their entire lengths, as ascertained by fluorescence *in situ* hybridization. Here, a probe to the 5S rDNA locus on chromosome V is shown in magenta, and a probe to the middle of chromosome IV (generated from cosmids C08F8, R07H5, M7, and F01G4) is shown in yellow. The images shown are projections through 3D data stacks encompassing whole nuclei. For each probe, the two homologous chromosomal targets are coincident or closely juxtaposed. Bar, 5 μm.

TABLE 2

Progeny viability following germline γ-irradiation

Genotype	γ-Irradiation	Total no. eggs laid	% inviable embryos	% adult survivors (95% C.I.)	Average no. of eggs/brood
Wild type	−	979	0.1	99.9 (±0.2)	196
Wild type	+	1289	15.0	85.0 (±1.9)	161
msh-5(me23)	−	1326	97.9	2.1 (±0.8)	166
msh-5(me23)	+	1701	99.2	0.8 (±0.4)	155

C.I., confidence interval.

ity of chromosome segregation. To investigate the role of *msh-5* in the recombination process, we similarly treated the germlines of *msh-5(me23)* hermaphrodites with γ-irradiation (5000 rads) and assessed the consequences for progeny viability, chiasma formation, and crossing over.

Whereas exposure of *spo-11* mutant hermaphrodites to γ-irradiation caused a 10- to 20-fold increase in the production of viable progeny (Dernburg *et al.* 1998), treatment of *msh-5* hermaphrodites did not increase progeny viability. Instead, irradiated *msh-5* hermaphrodites exhibited a modest decrease in the survivorship of their progeny embryos (Table 2). While this decrease in survivorship may be somewhat more severe than that observed for irradiated wild-type control hermaphrodites, it contrasts sharply with the elimination of survivorship that we observe in *mre-11* mutants, which are defective for repair of radiation-induced breaks (G. Chin and A. Villeneuve, unpublished results).

The failure of γ-irradiation to improve progeny viability suggested that radiation does not lead to efficient induction of chiasmata in *msh-5* mutant germ cells. We tested this directly by examining DAPI-stained oocyte nuclei in *spo-11* and *msh-5* mutant hermaphrodites at multiple time points following irradiation treatment (Table 3). Chiasmata were efficiently induced in the *spo-11* mutant: the vast majority of oocyte nuclei contained six bivalents, with 99% of homolog pairs held together by chiasmata at 18 hr following irradiation. In striking contrast, in the *msh-5(me23)* mutant most oocyte nuclei were indistinguishable from the unirradiated *msh-5* controls, containing 12 univalent chromosomes and no bivalents. Apparent bivalents were seen at a low frequency, with at most 4% of homolog pairs joined by chiasmata. Thus γ-irradiation-induced breaks do not bypass the requirement for *msh-5* for chiasma formation.

While chiasmata were not efficiently induced, the univalent chromosomes appeared morphologically intact after irradiation treatment. Once again, this contrasts with the results we obtained with the repair-defective *mre-11* mutants, where gross chromosomal abnormalities were observed following irradiation (G. Chin and A. Villeneuve, unpublished results). The high frequency of chiasmata induced in the *spo-11* mutant suggests that irradiation treatment generates an average of more than one DSB per chromosome (see materials and methods); thus the appearance of morphologically intact chromosomes suggests that to a large extent the *msh-5(me23)* mutant is able to repair radiation-induced DNA breaks. Further evidence that the *msh-5* mutant is competent to repair radiation-induced breaks comes from our observation that the percentage of male self-progeny does not decrease substantially following irradiation (40% males, $n = 184$ adult survivors); if chromosomes frequently suffered unrepaired lethal damage as a consequence of irradiation, we would expect a reduced recovery of viable males (relative to hermaphrodites) owing to hemizygosity for the X chromosome.

We also compared the *spo-11* and *msh-5(me23)* mutants for the frequency of crossovers induced by γ-irradiation in the *dpy-3 unc-3* interval on the X chromosome (Table 4). We previously showed that ~44% of oocyte-derived X chromosomes recovered following irradiation of *spo-11* germlines were recombinant in this interval, indicating that the apparent chiasmata induced at high efficiency in the *spo-11* mutant do indeed arise from crossover recombination events (Dernburg *et al.* 1998). In contrast, an estimated 6% of oocyte-derived X chromosomes recovered following irradiation of the *msh-5* mutant were recombinant. This result indicates that the vast majority (>85%) of crossover recombination events that can be induced by irradiation treatment depend on *msh-5* for their formation.

DISCUSSION

A conserved biological role for Msh5 proteins in promoting the crossover outcome of meiotic recombination events: We have shown here that *C. elegans msh-5* encodes a germline-specific member of the MutS protein family that is a crucial component of the nematode meiotic recombination machinery. Crossing over and chiasma formation are dependent on *msh-5* function and are severely reduced or eliminated in *msh-5* mutants. MSH-5 orthologs have been found to be important for normal meiosis in both yeast and mice (Hollingsworth *et al.* 1995; de Vries *et al.* 1999; Edelmann *et al.* 1999), but dramatic differences in their loss-of-function mutant

TABLE 3

Comparison of γ-ray-induced chiasma formation in *spo-11* and *msh-5* mutants

Genotype	γ-Irradiation	% bivalents[a] (no. of nuclei scored)		
		16 hr[b]	18 hr[b]	20 hr[b]
spo-11	−	0.6 (30)	ND	ND
spo-11	+	87.4 (115)	98.8 (55)	91.1 (56)
msh-5	−	1.5 (55)	ND	ND
msh-5	+	3.0 (211)[c]	4.2 (88)[d]	2.2 (59)[c]

The *msh-5(me23)* allele was used in this analysis. ND, not determined.

[a] % bivalents = (no. of observed bivalents)/(no. of potential bivalents), where the number of potential bivalents = 6 × (no. of nuclei scored). In some nuclei, individual univalents may lie too close together to be unambiguously resolved, and in such cases will be scored as bivalents.

[b] Time following irradiation treatment.

[c] Not significantly different from unirradiated 16-hr control ($0.1 < P < 0.5$).

[d] Borderline significance ($0.025 < P < 0.05$).

phenotypes had complicated efforts to deduce a specific conserved biological function for Msh5 proteins. Our analysis of both normal and artificially induced meiotic recombination using *C. elegans msh-5* mutants allows us to establish a clear conserved role for Msh5 proteins in promoting the crossover outcome of meiotic recombination events.

msh-5 is required not only for the formation of normal meiotic crossovers, but also for crossovers and chiasmata generated by artificially induced DNA breaks. Whereas treatment of *C. elegans* germ cells with γ-irradiation can bypass the requirement for the recombination-initiating enzyme SPO-11 for crossing over and chiasma formation (Dernburg *et al.* 1998), it does not bypass the requirement for MSH-5. We therefore conclude that *msh-5* cannot be required solely for the initiation step of meiotic recombination and suggest that it must function at some time after initiation.

We can also infer from our data that *msh-5* mutant germ cells are largely competent to repair DNA breaks induced during meiosis. The *msh-5* mutant does not exhibit the severe meiotic radiation sensitivity conferred by a defect in DSB repair, which completely abolishes progeny survivorship following germline irradiation and yields gross chromosomal abnormalities (G. Chin and A. Villeneuve, unpublished results). Instead, *msh-5* mutants exhibit only a modest reduction in progeny survivorship following germline irradiation, and the chromosomes emerge apparently intact at the end of meiotic prophase. These results suggest that a substantial fraction of the initiated recombination events must be completed in a way that restores an intact DNA duplex without allowing crossover formation. We suggest that these chromosomes may use their homologs as information donors in repair events that are resolved as noncrossovers, an outcome of meiotic recombination that is the natural alternative to crossover recombination; however, our data do not allow us to rule out the alterna-

TABLE 4

Comparison of γ-ray-induced recombination in *spo-11* and *msh-5* mutants

Parental genotype	γ-Irradiation	No. of X chromosomes	% recombinant X chromosomes[a]	Estimated % oocyte-derived recombinant X chromosomes[b]
spo-11; *dpy-3 unc-3*/++[c]	−	240	<0.5	
spo-11; *dpy-3 unc-3*/++[c]	+	215	22	44
msh-5; *dpy-3 unc-3*/++	−	387	0.5[d]	
msh-5; *dpy-3 unc-3*/++	+	272	3[d]	6

The *msh-5(me23)* allele was used in these experiments.

[a] Calculated as described in materials and methods.

[b] Because of the timing of irradiation treatment, induced crossover events will be restricted to oocyte meiosis (Dernburg *et al.* 1998). Since approximately half of X chromosomes recovered in viable progeny will be oocyte derived, we estimate the percent oocyte-derived recombinant X chromosomes to be twice the measured percentage of recombinant X chromosomes.

[c] *spo-11* data are from Dernburg *et al.* (1998).

[d] Significantly different, $0.010 < P < 0.025$ for χ^2 test.

tive possibility that repair is accomplished using sister chromatids rather than homologs as recombination partners.

The capacity of *msh-5* mutant germ cells to efficiently regenerate intact chromosomes once the recombination process has been initiated suggests that *msh-5* is not required for a process crucial to all recombination events, such as processing of DSB to yield a 3′ single-stranded region, or strand invasion. Rather our results suggest that *msh-5* functions specifically to promote the crossover outcome of meiotic recombination events. A similar conclusion has been drawn regarding the function of yeast Msh5, based on the observation that crossover frequencies are reduced by 50–70% in the *msh5* mutant, but gene conversion frequencies are unaffected (Hollingsworth *et al.* 1995).

There are several possible steps where Msh5 proteins might conceivably exert their conserved crossover-promoting capability. Several investigators have suggested that Msh5 and its heterodimer partner Msh4 may function at a step proximal to Holliday junction resolution (Ross-Macdonald and Roeder 1994; Hollingsworth *et al.* 1995; Pochart *et al.* 1997; Roeder 1997). We previously proposed a model in which accumulation of Msh4/Msh5 heterodimers might promote crossover resolution of double Holliday junctions by preventing junction sliding (which is proposed to be required to achieve a noncrossover outcome, Hastings 1988; McGill *et al.* 1989; Gilbertson and Stahl 1996), thereby constraining the intermediate to undergo resolution that results in crossing over (Zalevsky *et al.* 1999). This remains a viable hypothesis, but it is also worth considering models suggested by recent challenges to the assumption that crossover and noncrossover recombination products necessarily arise from common intermediates (Paques and Haber 1999); *e.g.*, Msh5 might act to stabilize an intermediate that is specific to the crossover pathway. Further, it is possible that Msh5 might act as early as the strand invasion step to promote a particular geometry of strand invasion that would impart an inherent bias toward the crossover outcome (Storlazzi *et al.* 1996).

"Rogue" *msh-5*-independent crossovers induced by irradiation? We note that while most potential crossover events that can be induced by the irradiation treatment require *msh-5* for their formation, we did recover recombinant X chromosomes at a significant frequency following irradiation of *msh-5(me23)* germlines. Since meiotic recombination occurs at the four-chromatid stage, a crossover frequency of 6% would normally indicate that 12% of X chromosome pairs had a chiasma in the assayed interval. This inferred chiasma frequency is higher than expected based on our direct cytological measurements of chiasma frequency in oocytes of irradiated *msh-5* hermaphrodites. Several possible explanations could reconcile this apparent discrepancy. Whereas the cytological assay scores all oocytes present at the time of the assay, the recombination assay scores only those chromosomes recovered in viable progeny; thus a bias favoring recovery of recombinant chromosomes would artificially inflate the measured crossover frequency. Another possibility takes into account the fact that the recombination assay assesses only X chromosome events, whereas the cytological assay considers the X chromosomes and the autosomes together; thus a differential effect of the irradiation treatment on the X chromosomes *vs.* the autosomes could account for the observed difference between the two assays. A third possibility is that some crossovers induced in the *msh-5* mutant background may not result in the formation of functional chiasmata if they initiate in an inappropriate spatial or temporal context. For example, if meiotic recombination events are normally initiated in proximity to chromosome cores, then events initiated near the apex of a chromatin loop might possibly be converted into a crossover at the DNA level without leading to a cytologically evident connection between the homologs.

Regardless of the source of the apparent discrepancy between the infrequent events detected by genetic and cytological assays, the basic conclusion from this set of experiments is unaffected: the efficient conversion of artificially induced DNA breaks into crossovers and functional chiasmata seen in the absence of *spo-11* occurs by a process that is dependent on *msh-5*.

If *msh-5* is dispensible for progression through meiosis in *C. elegans*, why is its ortholog required in mice? Apart from the severe defect in crossover recombination and consequent lack of chiasmata, progression through meiotic prophase is largely unperturbed in both of our *msh-5* mutants. Chromosome morphology appears normal, there is a typical distribution of nuclei at all cytologically distinguishable stages, and homologous chromosomes efficiently achieve full intimate pairing and alignment. Further, most germ cells normally destined to survive and complete meiosis do indeed survive in the *msh-5* mutant and go on to complete meiosis and gametogenesis and to produce zygotes.

The ability of *C. elegans msh-5* mutant germ cells to survive and progress through meiosis contrasts sharply with the fate of germ cells in *Msh5*−/− mice (de Vries *et al.* 1999; Edelmann *et al.* 1999). Mouse *Msh5*−/− germ cells rarely progress beyond the zygotene stage, frequently exhibit a lack of synapsis between homologs and/or abnormal synapsis between nonhomologous chromosomes, and ultimately undergo apoptosis, resulting in severe hypogonadism and sterility. On the basis of these phenotypes, it has been suggested that murine Msh5 functions to promote pairing and/or synapsis of homologous chromosomes. However, the absence of a similar requirement for *C. elegans msh-5* indicates that such a role is not a conserved feature of metazoan meiosis. The evolutionary relationships among MutS family members have been analyzed in great detail, and the orthologous relationship between

these proteins is not in dispute (Eisen 1998). Has murine Msh5 modified its biological role, or acquired an additional function beyond its conserved function in crossover recombination? Or does the mouse phenotype instead represent a difference in the physiological consequences of lacking a protein with the conserved biological role assigned to the Msh5 family based on analysis in worms and yeast?

The relationship between recombination and synapsis is not well established in mice, but there is evidence to suggest that some events in recombination may be required for normal homologous synapsis and/or for progression through meiotic prophase (Pittman *et al.* 1998; Yoshida *et al.* 1998). Thus it is possible that the synapsis/progession defects in the mouse *Msh5* mutants are a consequence of a primary defect in the crossover recombination process. In *C. elegans*, complete homologous synapsis is achieved even in the absence of recombination initiation (Dernburg *et al.* 1998), so it is not surprising that a defect at a later step (particularly one that allows repair of initiated events) would have no apparent consequences for synapsis and would allow meiotic progression.

We would like to entertain an alternative (admittedly *ad hoc*) hypothesis, that mouse germ cells might have a mechanism for assessing whether key components of the meiotic machinery are present in the cell before proceeding to complete meiosis and gametogenesis. This notion differs from the traditional idea of a checkpoint as a mechanism that monitors completion of a process, the presence of defective intermediates, or both (Weinert and Hartwell 1989). A capacity to take inventory before proceeding might be particularly useful in placental organisms, where gestation of aneuploid zygotes can exact a substantial energetic toll.

***msh-5* dependence of artificially induced crossovers: implications for the mechanism of meiotic recombination:** We have shown here that most of the crossover recombination events initiated by artificially induced breaks proceed through the normal recombination pathway, requiring the normal meiotic recombination machinery. A similar conclusion has been reached recently based on a parallel line of investigation carried out in budding yeast. Malkova *et al.* (1996) had shown previously that meiotic recombination events could be initiated by DSBs generated by the HO endonuclease (Kostriken *et al.* 1983; Kostriken and Heffron 1984) expressed under a meiosis-specific promoter. They now find that HO-induced recombination events behave much like normal meiotic recombination events based on several criteria, including frequent association with crossing over and dependence of half of the crossovers on *MSH4* (A. Malkova and J. Haber, personal communication). Together the nematode and yeast studies suggest that there is nothing inherently special about the DSBs generated by SPO-11/Spo11p that is a prerequisite for entering the meiotic recombination pathway. Although Spo11 proteins apparently generate breaks by a topoisomerase-like mechanism involving covalent attachment to the DNA, this mode of break induction is not necessary for subsequent steps in recombination to proceed. Moreover, there is no evident requirement for a preinitiation complex associated with a break.

Further, our finding that most crossovers and chiasmata generated by artificially induced breaks require the normal *msh-5*-dependent meiotic recombination pathway places constraints on when crossover bias is established in meiosis. Previous data did not allow us to infer when in the recombination process the crossover decision is initially made. Thus it was possible that commitment to the crossover outcome might be made at or prior to initiation, at the strand invasion step, or in the formation, stabilization, or imposition of constraints on subsequent intermediates. The fact that artificially induced breaks could bypass the requirement for SPO-11 for formation of crossovers and chiasmata had suggested that the crossover decision is likely made after the initiation step, but this conclusion was contingent upon knowing that the induced crossovers were generated by the normal meiotic recombination machinery. With this knowledge now in hand, we can conclude that there is no requirement to establish a bias toward the crossover outcome at or prior to the initiation step of meiotic recombination. Thus the decision regarding which initiated events will become crossovers is likely made at a later step in the recombination process.

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