

Distinct Functions of MLH3 at Recombination Hot Spots in the Mouse

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ABSTRACT

The four mammalian MutL homologs (MLH1, MLH3, PMS1, and PMS2) participate in a variety of events, including postreplicative DNA repair, prevention of homeologous recombination, and crossover formation during meiosis. In this latter role, MLH1–MLH3 heterodimers predominate and are essential for prophase I progression. Previous studies demonstrated that mice lacking *Mlh1* exhibit a 90% reduction in crossing over at the *Psb9* hot spot while noncrossovers, which do not result in exchange of flanking markers but arise from the same double-strand break event, are unaffected. Using a PCR-based strategy that allows for detailed analysis of crossovers and noncrossovers, we show here that *Mlh3*^{-/-} exhibit a 85–94% reduction in the number of crossovers at the *Psb9* hot spot. Most of the remaining crossovers in *Mlh3*^{-/-} meocytes represent simple exchanges similar to those seen in wild-type mice, with a small fraction (6%) representing complex events that can extend far from the initiation zone. Interestingly, we detect an increase of noncrossovers in *Mlh3*^{-/-} spermatocytes. These results suggest that MLH3 functions predominantly with MLH1 to promote crossovers, while noncrossover events do not require these activities. Furthermore, these results indicate that ~10% of crossovers in the mouse are independent of MLH3, suggesting the existence of alternative crossover pathways in mammals.

MEIOTIC recombination between homologous chromosomes during prophase I can result in both crossover (CO) and noncrossover (NCO) products. Most, if not all, of these events are initiated by the same double-strand break (DSB) event induced by SPO11, a conserved protein sharing sequence similarities with the catalytic subunit of TopoVI, a type II DNA topoisomerase from archae (reviewed in KEENEY and NEALE 2006), but are processed through distinct and highly conserved molecular pathways, the details of which remain to be elucidated fully in mammals. What is clear from studies in a number of species is that the frequency and distribution of CO events are tightly controlled in prophase I, and that this control is essential for the accurate reductional segregation of chromosomes at the first meiotic division.

Studies in *Saccharomyces cerevisiae* have shown that CO and NCO events result from two distinct pathways that differ in their specific DNA intermediates and functional requirements (ALLERS and LICHTEN 2001; HUNTER and KLECKNER 2001). In particular, a specific group of proteins composing the ZMM family, encoded by the yeast *ZIP1*, *ZIP2*, *ZIP3*, *ZIP4*, *MSH4*, *MSH5*, and *MER3* genes, acts early in the formation of most CO but

is not required for NCO formation (BORNER *et al.* 2004). The yeast MutL homologs, Mlh1 and Mlh3, are also necessary for CO formation but act at a later step, presumably after the formation of the double-Holliday-junction intermediate (HUNTER and BORTS 1997; WANG *et al.* 1999; ARGUESO *et al.* 2004). In mammals, MSH4 and MSH5 are thought to be required at an early step of meiotic recombination, given that mutant mice are defective in synapsis and that the proteins localize along chromosome axes as early as leptoneuma in both males and females (KNEITZ *et al.* 2000; LENZI *et al.* 2005). These proteins have also been proposed to have a later action on CO formation on the basis of their binding activities to double Holliday junctions (SNOWDEN *et al.* 2004) and the colocalization of MSH4 with MLH1 at midpachynema (SANTUCCI-DARMANIN *et al.* 2000).

Mammalian MLH1 and MLH3 proteins also appear to be involved and required for CO formation on the basis of localization studies and the phenotypes of *Mlh1* and *Mlh3* mutant mice (KOLAS and COHEN 2004). At mid- to late pachynema, these proteins colocalize (KOLAS *et al.* 2005) at the future sites of chiasmata (MARCON and MOENS 2003). Both *Mlh1* and *Mlh3* nullizygous mice show a strong defect in chiasma formation in both sexes (BAKER *et al.* 1996; EDELMANN *et al.* 1996; LIPKIN *et al.* 2002; KAN *et al.* 2008). However, recent analysis has revealed different properties of these two MutL homologs: MLH3 accumulates at nascent CO sites prior to MLH1 in males and can continue to do so in mice lacking a functional *Mlh1* gene (LIPKIN *et al.* 2002; KOLAS

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and COHEN 2004). Conversely, MLH1 requires MLH3 preloading at MSH4–MSH5 sites since no MLH1 is localized to meiotic chromosomes in spermatocytes from *Mlh3*^{-/-} mice (KOLAS and COHEN 2004; KOLAS *et al.* 2005). At the same time, electron-dense meiotic nodules, the sites at which recombination events are taking place, persist on meiotic chromosomes from *Mlh1*^{-/-} mice but not on chromosomes from *Mlh3*^{-/-} mice (LIPKIN *et al.* 2002). Direct analysis at the DNA level has shown that MLH1 is required for most but not all CO and not required for NCO formation (GUILLON *et al.* 2005).

To examine the role of MLH3 directly on meiotic recombination events, we examined the CO and NCO frequencies at the well-characterized *Psmb9* recombination hot spot on mouse chromosome 17, using a strategy that allows for detailed analysis of CO and NCO products at this locus (GUILLON and DE MASSY 2002; GUILLON *et al.* 2005; BAUDAT and DE MASSY 2007b). Our analyses of recombination in male and female germ cells demonstrate that, similar to *Mlh1*^{-/-} mice, *Mlh3* null animals exhibit an 85–94% reduction in the number of crossings over at the *Psmb9* hot spot, while the respective NCO frequencies remain unchanged. Most remaining crossovers in *Mlh3*^{-/-} spermatocytes represent simple exchanges similar to those seen in wild-type mice. However, a small fraction (6%) of residual COs are complex events with exchange points far from the initiation zone and similar in profile to the residual CO events observed in *Mlh1*^{-/-} spermatocytes. A significant increase of NCO frequencies was observed in spermatocytes from *Mlh3*^{-/-} mice, potentially indicating a channeling of some CO intermediates into NCO products. These results suggest that MLH3 functions predominantly with MLH1 to promote crossing over and that, together, this heterodimer governs ~90% of all CO events in mouse meocytes.

MATERIALS AND METHODS

Generation and maintenance of hybrid mice: Mice were housed in the Cornell University Animal Facility (Ithaca, NY) and all procedures utilizing these animals were reviewed and approved by the Cornell University Institutional Animal Care and Use Committee. Mice were maintained on standard laboratory chow under controlled conditions of light and temperature. The generation of *Mlh3* mutant mice has been described previously (LIPKIN *et al.* 2002). Mice heterozygous at the *Psmb9* (*wm7/b*) locus and carrying one or two mutant alleles of *Mlh3* (*Mlh3*^{+/+}, *Mlh3*^{+/-}, or *Mlh3*^{-/-}) were generated by crossing the B10.A (R209) strain carrying the *wm7* haplotype (named R209, B10 background) and the *Mlh3*^{+/-} strain (B6 background). Progeny of *Mlh3*^{+/-} mice (B6 background) crossed with *Mlh3*^{+/-} *wm7/wm7* (B6/B10 mixed background) mice were analyzed, providing *Mlh3*^{-/-}, *Mlh3*^{+/-}, and *Mlh3*^{+/+} littermates. Testes were dissected from mice at 11, 13, 15, 17, and 19 days postpartum (pp), while ovaries were taken from mice at 1 day pp. Genomic DNA preparations were used for subsequent PCR analysis of CO and NCO products.

Detection and mapping of COs and NCOs: COs were detected in two ways: (1) direct selection with two rounds of allele-specific PCR with primers specific for B6 on one side and for R209 on the other side of the hot spot (GUILLON and DE MASSY 2002) and (2) by a protocol allowing the detection of NCO in parallel to CO. In this protocol, B6-R209 (BR) COs were detected using in the first round of amplification (PCR I) a B6 allele-specific forward primer and a universal reverse primer. In the secondary amplification reactions (PCR II) a B6 allele-specific forward primer was used in combination with an R209 allele-specific reverse primer. Using the same PCR I products as substrates, an alternative secondary amplification is performed to detect NCO products with a gene conversion at the *BsrFI* site. This is achieved using an R209 *BsrFI*-specific forward primer with a B6 allele-specific reverse primer. Although the *BsrFI* site is located near the center of the hot spot, given the short sizes of gene conversion tracts among NCOs, we estimate that those covering *BsrFI* represent less than half of the total (F. BAUDAT and B. DE MASSY, personal communication). For direct selection of COs, PCR I can be performed on pools of testis DNA containing up to 10,000 amplifiable molecules, whereas for the parallel detection of COs and NCOs, PCR I is performed on pools of testis DNA containing up to 400 amplifiable molecules. Both methods gave similar values of CO frequencies, which were therefore determined by averaging independent experiments using either protocol. For mapping analysis, the CO molecules were obtained from direct selection, using at the second PCR forward and reverse primers located at positions 402 and 4315, respectively, therefore leading to the amplification of a 4-kb product (see Figure 3). Details of the procedures and primer sequences are as described in GUILLON *et al.* (2005). A small-scale experiment on 24 pools at all time points in wild type and *Mlh3*^{-/-} indicated that frequency of RB crossover molecules was similar to that of BR molecules as expected (data not shown). No significant difference of CO or NCO frequencies could be detected between +/+ and +/- mice. COs were first mapped by RFLP at 10 sites in the amplified region. Those showing unexpected patterns were sequenced over the whole 4-kb region. NCOs were tested for the presence of *StyI* and *SphI* sites and were sequenced upstream of the *BsrFI* site. Those showing single-site conversions at *BsrFI* were sequenced downstream of this site. Additional CO events were selected and analyzed from *Mlh1*^{-/-} mice for mapping analysis.

RESULTS

A specific reduction of CO frequencies both in spermatogenesis and in oogenesis in *Mlh3*^{-/-} mice: Using a strategy and protocols similar to those developed for the analysis of recombination events in *Mlh1*^{-/-} mice (GUILLON *et al.* 2005), we have measured the frequency of COs and NCOs at the *Psmb9* recombination hot spot. The general strategy is as follows: mice heterozygous at *Psmb9* (*wm7/b*) and homozygous mutant at *Mlh3* (*Mlh3*^{-/-}) were generated along with *Mlh3*^{+/-} or *Mlh3*^{+/+} controls. Analyses in males were performed on genomic DNA extracted from testes of mice from 11 to 19 days old, before the massive induction of apoptosis occurring at metaphase I (LIPKIN *et al.* 2002). Analyses in females were performed on ovaries from newborn mice. CO and NCO molecules were detected by allele-specific PCR on small pools of genomic DNA. Two protocols were used, one allowing the detection of CO products only and one

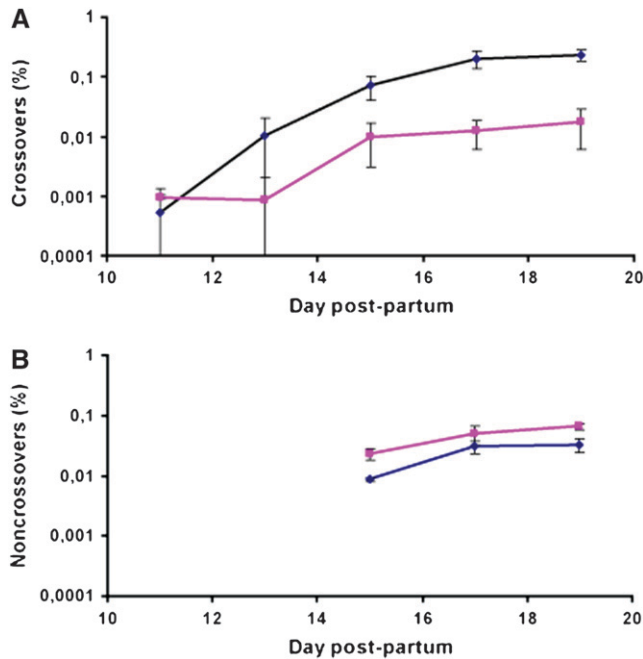


FIGURE 1.—CO and NCO frequencies in *Mlh3*^{-/-} mice. (A) CO frequencies in wild-type (wt) (+/+ or +/-) and *Mlh3*^{-/-} mice during the first wave of spermatogenesis. Values correspond to twice the frequency of BR recombinant molecules and were the average from two measurements at 11 and 13 days and from five to eight measurements at 15, 17, and 19 days. Frequencies are given \pm standard error (SE) (blue diamonds, wt; pink squares, *Mlh3*^{-/-}). (B) NCO frequencies in wt (+/+ or +/-) and *Mlh3*^{-/-} mice during the first wave of spermatogenesis. Values correspond to the frequencies of NCOs with a gene conversion selected at *BsrFI* on the B6 parental genome and were the average of two, four, and three measurements at 15, 17, and 19 days, respectively. Each frequency measurement was obtained from allele-specific PCR on 96 pools. Frequencies are given \pm SE (blue diamonds, wt; pink squares, *Mlh3*^{-/-}).

allowing the parallel detection of COs and NCOs. The allele-specific PCR strategy we have used allows the recovery of all CO molecules in the 4-kbp interval tested but only a fraction of NCOs, those with gene conversion tracts covering the central *BsrFI* site (see MATERIALS AND METHODS).

During meiosis in males, the frequency of CO events was found to be 85–94% lower in spermatocytes from *Mlh3*^{-/-} males compared to wild type at all time points during the first wave of spermatogenesis where a significant number of events could be recovered, *i.e.*, at 13, 15, 17, and 19 days. This reduction in CO content was statistically significant at 15, 17, and 19 days, using a one-tailed *t*-test (*P*-values of 0.0455, 0.0004, and 0.0004 at 15, 17, and 19 days, respectively). The kinetics of CO formation were similar in wild-type and *Mlh3*^{-/-} spermatocytes, with a plateau reached at 17 days when cells progressing through the first wave of meiosis reach the end of the pachytene stage (Figure 1A). The analysis of events in female meiosis also indicated a 94% reduction in CO frequencies (one-tailed *t*-test, *P* < 0.0001; Table 1).

TABLE 1

CO and NCO frequencies in ovaries from wt (+/+ and +/-) and *Mlh3*^{-/-} mice

	CO frequencies (%)	Standard error (%)	No. of events
<i>Mlh3</i> ^{-/-}	0.0087	0.0103	8
<i>Mlh3</i> ^{+/+} and <i>Mlh3</i> ^{+/-}	0.140	0.044	108
Ratio wt/mutant	16		
	NCO frequencies (%)	Standard error (%)	No. of events
<i>Mlh3</i> ^{-/-}	0.051	0.035	35
<i>Mlh3</i> ^{+/+} and <i>Mlh3</i> ^{+/-}	0.046	0.012	45
Ratio wt/mutant	0.9		

CO frequencies reported are two times the frequencies of BR recombinant molecules measured. The NCO frequencies are NCOs selected at *BsrFI* on the B6 chromosome. The numbers of recombinant events detected are provided.

In striking contrast, no reduction in NCO frequencies in *Mlh3*^{-/-} mice was observed either in spermatocytes or in oocytes (Figure 1B and Table 1). In fact, we detected an increase of 67–171% in NCO rates in *Mlh3*^{-/-} spermatocytes between days 15 and 19 postpartum. These differences between wild-type and *Mlh3*^{-/-} mice are statistically significant (one-tailed *t*-test, *P*-values of 0.0291, 0.0386, and 0.0036 at 15, 17, and 19 days, respectively). The small difference between wild-type and *Mlh3*^{-/-} mice observed in oocytes was not significant, however (*P* = 0.43963).

Properties of residual crossovers in the absence of MLH3: We have mapped the positions of exchange points among 122 and 308 CO products in wild-type and *Mlh3*^{-/-} mice, respectively. In wild-type mice, all COs but one were simple exchange products with an exchange point in the interval between *TaqI* and *Eco57I*. The distribution of exchange points was similar to that in previous analyses (GUILLON and DE MASSY 2002; GUILLON *et al.* 2005).

In *Mlh3*^{-/-} males, most CO products (94%, 290/308) have a structure similar to wild type, with one exchange point within the *TaqI* and *Eco57I* interval and with the highest density in the *BsrFI*–*StyI* interval. The distribution is globally similar to that observed in wild type, as in previous analyses, but with a higher density in the *BsrFI*–*StyI* interval, suggesting that the conversion tracts associated with these CO events are shorter (Figure 2). The distributions of wild-type and *Mlh3*^{-/-} COs are indeed statistically different (chi square, *P* = 0.028). However, the remaining fraction of CO molecules (6%, 18/308) in *Mlh3*^{-/-} males displays unusual structures, and these are not observed in wild-type control males. Among these unusual recombinant molecules, we could identify three types of molecules: COs with one single exchange point located distal to the hot spot, COs with multiple exchange points (with mosaic structure), and

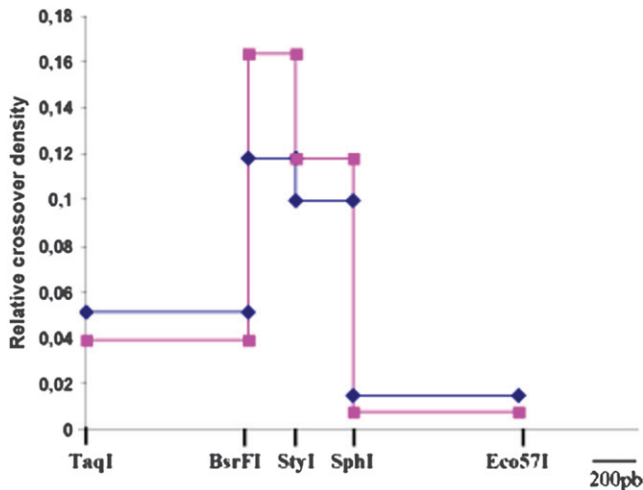


FIGURE 2.—Mapping of exchange points among simple CO products in *Mlh3*^{+/+} and *Mlh3*^{-/-} mice. CO products were recovered from genomic DNA from the testes of 17- and 19-day pp mice. COs were obtained by amplification of a 4-kb interval by primers located at positions 402 and 4315 (see Figure 3). Only those with a single exchange in the *TaqI*–*Eco57I* interval are integrated in this graph. The relative crossover density is the proportion of COs in each interval (relative to all COs between *TaqI* and *Eco57I*) divided by its length. Intervals are defined by RFLP sites *TaqI*, *BsrFI*, *StyI*, *SphI*, and *Eco57I* (blue diamonds, wt; pink squares, *Mlh3*^{-/-}).

COs containing mixed-strand information (Figure 3). These CO products with mixed strands could be either molecules containing heteroduplex DNA or a mixture of a CO with a simple exchange and a CO with a mosaic structure. This second possibility is statistically compatible with the number of CO events per pool in these experiments (supplemental Table 1). In any case, these observations indicate a modification of the processing of recombination intermediates in *Mlh3*^{-/-} males. The mapping of these products suggests that recombination intermediates extend farther away from the initiation zone than those generated in wild-type meocytes and/or that the repair of heteroduplex structures is altered in the absence of MLH3 protein. Although a qualitatively similar observation was made in *Mlh1*^{-/-} males (GUILLON *et al.* 2005), we noted that the relative proportion of COs with a single distal exchange point was higher in the absence of MLH3 as compared to that observed by Guillon *et al.* in the absence of MLH1. To more accurately compare the two mutant strains, we repeated these CO analyses from *Mlh1*^{-/-} spermatocytes using identical conditions to, and in parallel with, our studies in *Mlh3*^{-/-} spermatocytes. Although the relative proportion of COs with a distal exchange point was higher in *Mlh3*^{-/-} spermatocytes compared to *Mlh1*^{-/-} spermatocytes (50–55% *vs.* 17–30%), the difference is not statistically significant (supplemental Figure 1 and supplemental Table 1). It appears therefore that the absence of MLH1 or MLH3 has similar consequences for residual CO processing.

No detectable change in NCO maps in *Mlh3*^{-/-} mice:

The NCO products were selected at the *BsrFI* site where flanking markers are at 329 and 214 bp to the left and right, respectively. In both wild-type and *Mlh3*^{-/-} spermatocytes most of the NCO products selected at *BsrFI* have a conversion tract including only the *BsrFI* marker (95 and 88%, respectively). Some rare products were observed to coconvert a marker flanking *BsrFI*. In addition, in spermatocytes and oocytes from *Mlh3*^{-/-} mice, some rare molecules were found to have mosaic structures or mixed strands (Figure 4). However, no significant difference in the frequencies of these different products could be detected between wild-type and *Mlh3*^{-/-} male or female mice.

DISCUSSION

The ability to detect recombinant molecules directly by allele-specific PCR has allowed us to analyze, at the DNA level, the effect of MLH3 deletion on meiotic recombination in mice, in terms of both frequency and mapping of CO and NCO events at the *Psmb9* hot spot. Our results show a differential effect between COs and NCOs with a strong reduction of CO frequency (85–94%) whereas NCO frequency is not affected or slightly increased. The analysis of COs detected in the absence of MLH3 reveals a fraction of events that differ in the location of exchange points compared to wild-type meocytes. This suggests that MLH3 plays a role in the processing of recombination intermediates.

The role of MLH3 and MLH1 in CO formation: The reductions of CO frequencies during the first wave of spermatogenesis and during oogenesis of *Mlh3*^{-/-} mice, demonstrated herein, reveal an essential role for MLH3 for CO formation. This finding is consistent with the reduction of chiasmata in *Mlh3*^{-/-} males (LIPKIN *et al.* 2002) and implies that the effect on chiasma is not due to a defect in chiasma stabilization but results directly from a defect in CO formation. The quantitative effect on CO formation is identical to that previously described for *Mlh1*^{-/-} mice (GUILLON *et al.* 2005), a result consistent with the similar defect on chiasma formation also described in these two mutants. This suggests that both proteins act in the same pathway with respect to CO formation, which is supported by immunological studies that have shown the colocalization of MLH1 and MLH3 at pachynema (KOLAS *et al.* 2005) and at sites of chiasmata (MARCON and MOENS 2003). At the same time, however, subtle differences in MLH1 and MLH3 focus dynamics indicate that the loss of either gene product might not necessarily have the same effect on CO frequency in *Mlh1*^{-/-} and *Mlh3*^{-/-} animals. First, in males MLH3 foci form earlier (early pachynema) and possibly in greater initial numbers as compared to MLH1; second, meiotic nodules observed by electron microscopy are still detected in *Mlh1*^{-/-} males but are

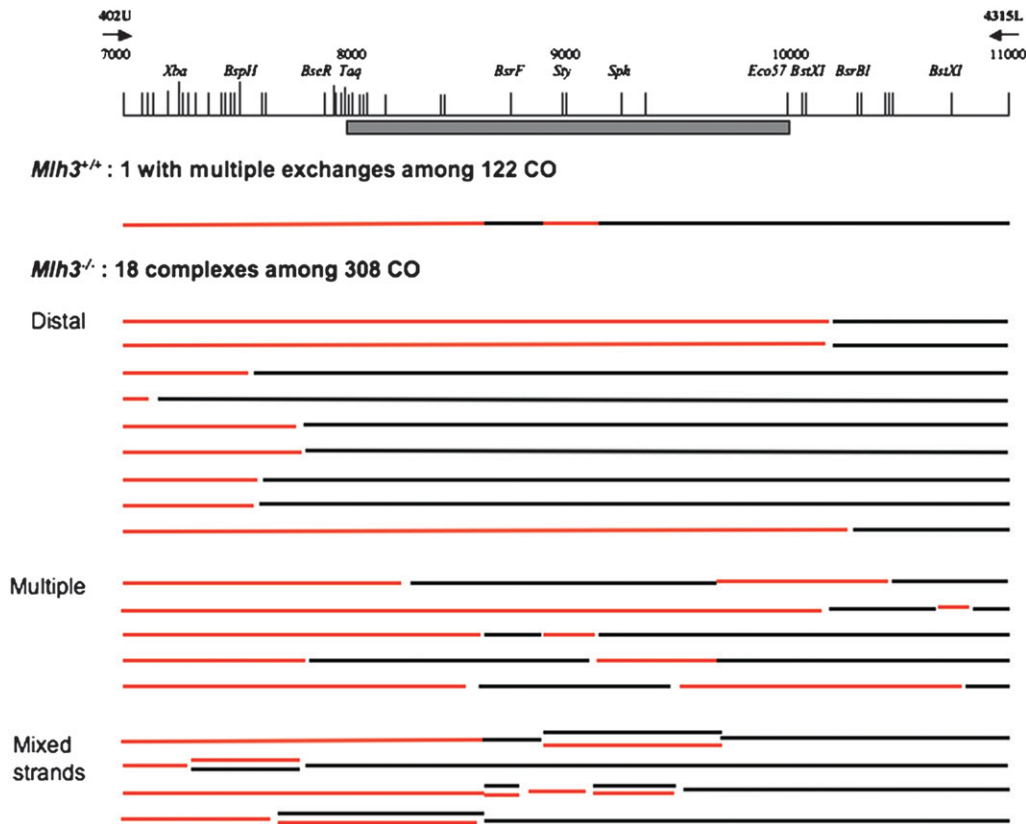


FIGURE 3.—Mapping of exchange points among complex CO products. One hundred twenty-two COs and 308 COs were mapped in wt and *Mlh3*^{-/-} (respectively) from genomic DNA of day 17 and day 19 pp testes. They were obtained as 4-kb molecules amplified with primers at positions 402 and 4315. The hot spot, identified by a shaded bar, is located between the *TaqI* and *Eco57I* sites and centered in the *BsrFI*–*StyI* region. Red and black lines represent B6 and R209 haplotypes, respectively. Those molecules with unusual positions of exchange points, *i.e.*, more than one exchange point or an exchange point outside the *TaqI*–*Eco57I* interval, were sequenced and divided into three categories: molecules with a distal exchange point, multiple exchanges, and mixed strands.

absent in *Mlh3*^{-/-} males (LIPKIN *et al.* 2002); and third, the presence of MLH1 foci depends on preloading of MLH3, whereas a significant number of MLH3 foci are still detected in *Mlh1*^{-/-} (KOLAS *et al.* 2005). These differences in cytological observations compared to the similar defect in CO formation in *Mlh3*^{-/-} and *Mlh1*^{-/-} males suggest that the persistent CO events observed at the *Psmb9* locus in *Mlh1*^{-/-} males are not due to residual MLH3 activity and that neither MLH1 nor MLH3 can function independently of each other in the context of meiotic recombination events.

The localization of MLH1 and MLH3 to meiotic nodules is independent of the somatic repair MutS homolog heterodimers, MutS α (MSH2/MSH6) or MutS β (MSH2/MSH3) and instead operates through interactions with the meiosis-specific MutS complex, MutS γ , that consists of MSH4 and MSH5. This suggestion is supported by the interaction between human MSH4 and MLH3 (SANTUCCI-DARMANIN *et al.* 2002) and by the interaction and colocalization of MSH4 and MLH1 in mice (SANTUCCI-DARMANIN *et al.* 2000) as well as in humans (OLIVER-BONET *et al.* 2005). According to *in vitro* analysis of mismatch repair activity, MutL proteins are thought to be adaptors able to transduce signals, possibly involving protein conformational changes. MLH1/MLH3 might serve to modulate the accumulation of MSH4/MSH5 sliding clamps by stabilizing these structures and/or by facilitating the sliding

of MSH4/MSH5 along the chromosomes, thereby facilitating the loading of additional factors at the Holliday-junction structure and biasing these sites toward a CO fate. If recombination intermediates are not converted into COs in *Mlh1*^{-/-} and *Mlh3*^{-/-} meocytes, at least two outcomes can be envisioned: either they are left unresolved or they are processed into alternative products. We suggest that a fraction of these intermediates are actually resolved into NCOs with the dissolution activity of BLM-TOP3 α (WU and HICKSON 2003). The increase of NCO frequency detected in *Mlh3*^{-/-} spermatocytes is consistent with this hypothesis. At the quantitative level, we cannot determine what fraction of COs is channeled into NCOs, as this would require knowledge of the gene conversion tract length and distribution among the NCO molecules. In addition, although the effect of unresolved intermediates on meiotic prophase progression is unknown, the analysis of RAD51C hypomorph mutant mice has led to the suggestion that unresolved intermediates would induce chromosome breakage (KUZNETSOV *et al.* 2007), a phenotype not observed in either *Mlh1*^{-/-} or *Mlh3*^{-/-} mice.

A residual activity of CO formation in the absence of MLH3: Our results also clearly show that a residual level of crossing over, ~10% of wild type, occurs in *Mlh3*^{-/-} mice. If this subset of events also occurs in wild-type meocytes, one implication is that MLH1/MLH3 foci would not be expected to mark all sites of COs. We note,

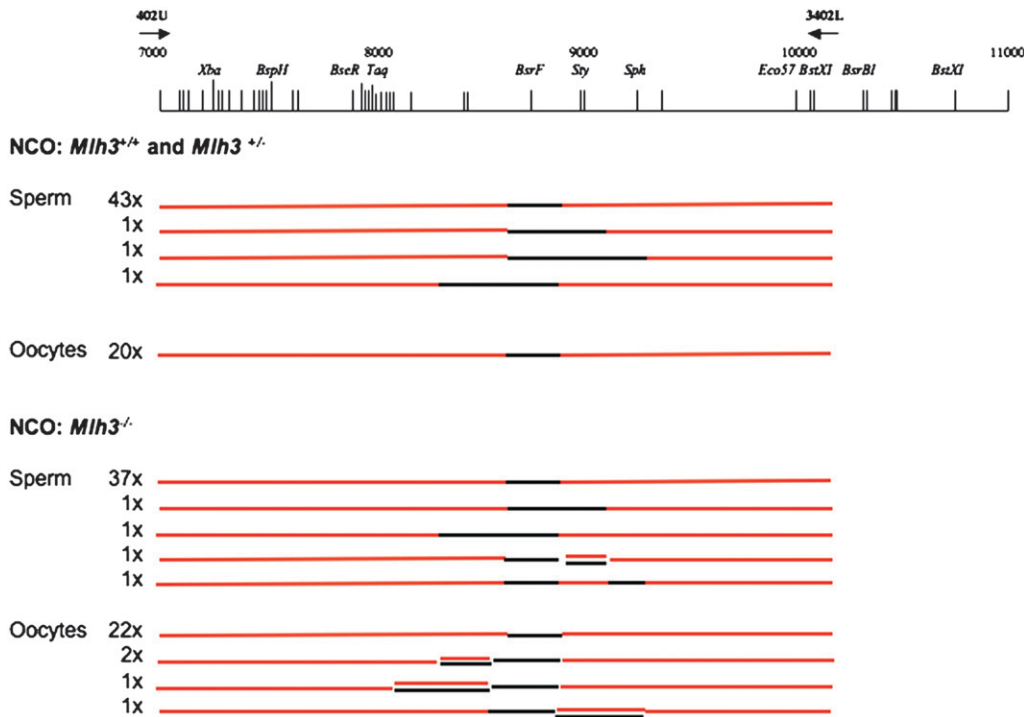


FIGURE 4.—Mapping of NCO products in *Mlh3*^{+/+} and *Mlh3*^{-/-} mice. Both left and right sides of independent NCO events with a conversion at *BsrFI* were selected by allele-specific PCR and sequenced. Most events are converted at the *BsrFI* marker only. DNA was extracted from either newborn ovaries or testes of day 17 and day 19 pp wild-type and *Mlh3*^{-/-} mice.

in fact, that a small difference in terms of genetic map length is observed when estimated from MLH1 foci or from genetic mapping (BAUDAT and DE MASSY 2007a). Despite this, however, it is clear that the major pathway for CO formation in mouse is MLH3 (and MLH1) dependent. In *S. cerevisiae*, it has been established that most of the Mlh1-independent COs result from the Mus81/Mms4 pathway (HOFFMANN *et al.* 2003; ARGUESO *et al.* 2004; HOLLINGSWORTH and BRILL 2004), while in *Schizosaccharomyces pombe*, all COs require Mus81/Mms4 (SMITH *et al.* 2003). In the case of the latter organism, single, not double, Holliday junctions have been shown to be intermediates of meiotic recombination (CROMIE *et al.* 2006), and these are readily cleaved by the Mus81/Mms4 proteins *in vitro* (BODDY *et al.* 2001; OSMAN *et al.* 2003). Whether an analogous, “alternate” pathways exists in mice remains to be seen. The normal fertility of MUS81 mutant mice (HOLLINGSWORTH and BRILL 2004; DENDOUGA *et al.* 2005) is compatible with the idea that any MUS81-driven recombination events would concern only a small fraction of CO events and/or would be elicited only in the absence of appropriate MLH1/MLH3 activity.

The mapping of the remaining COs that occur in *Mlh3*^{-/-} meocytes also provides additional information about COs generated by the MutLγ-independent pathway. Two categories of CO products are detected: the majority (94%) have a single exchange point with a distribution similar to that seen in meocytes from wild-type animals, and a smaller population (6%) have atypical exchange points (discussed below). Interestingly, the majority class shows a slight difference in

terms of distribution from that of wild type, with a narrower clustering of exchange points around the predicted region of initiation, suggesting that the mean length of the associated conversion tract is smaller. A similar effect was observed in *Mlh1*^{-/-} animals (GUILLON *et al.* 2005). This suggests a difference between the two pathways (MutLγ dependent or independent), either in the formation or in the processing of intermediates. Whether the resolution of this MutLγ-independent class of COs is also independent of MutSγ remains to be seen. What is clear, however, is that the absence of mismatches among the COs recovered in the majority class indicates that the heteroduplex DNA generated during the formation of these COs have been repaired in the absence of MLH3. Given that a similar conclusion was reached from the analysis of *Mlh1*^{-/-} male mice, if a MutL activity is involved in mismatch repair, it is more likely to involve PMS2 assuming it can act with either MLH1 or some other partner. *In vitro* biochemical data indicate that PMS2 and MLH3 do not form a complex with each other, ruling out a role for MLH3 in heteroduplex repair, even in *Mlh1*^{-/-} animals.

Exchanges at distal locations in the absence of MLH3: The detailed mapping of CO products from *Mlh3*^{-/-} males reveals a small fraction (6%) of products with unusual properties, not seen in wild-type meocytes, but also detected in the absence of MLH1. Fifteen of 18 of these CO events exhibit an exchange point located >1 kb away from the region of initiation. Among these distal events, 9 have a single exchange point. Such products could be derived from a DSB repair event exemplified in Figure 5, either resulting from intermedi-

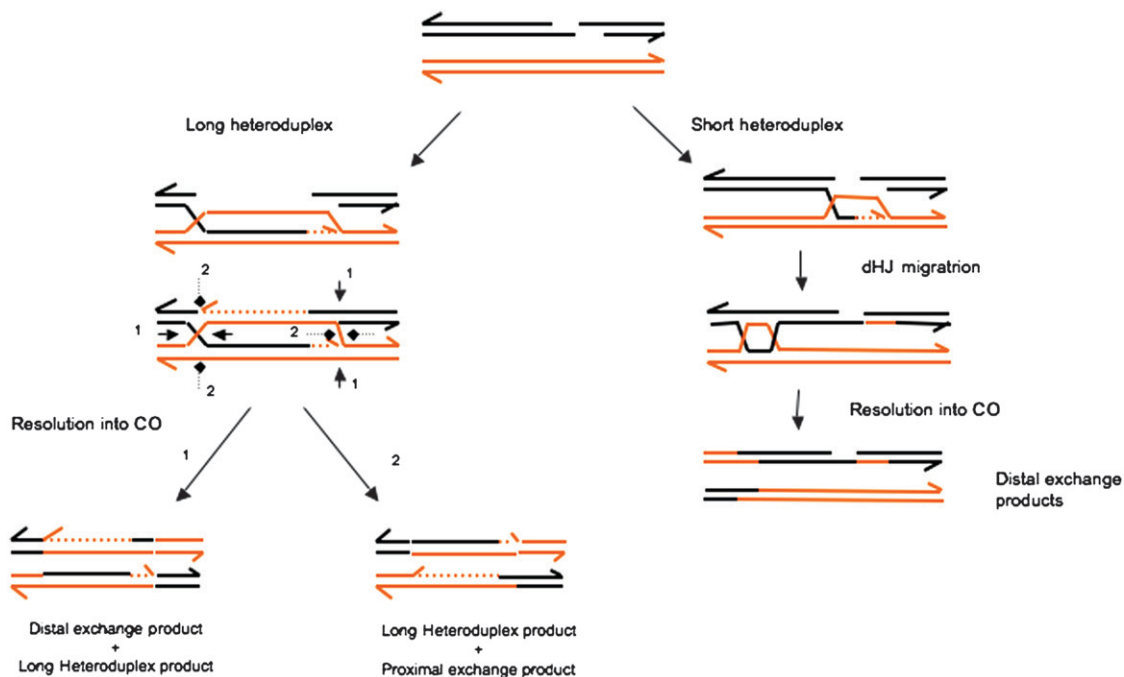


FIGURE 5.—Alternative ways to generate distal exchange points on CO products. Two alternative ways are considered for the formation of COs with a distal exchange point without long heteroduplexes. One implies the formation of a long asymmetric heteroduplex and the other the migration of a double Holliday junction. In the first alternative, products differ depending on the directionality of resolution. Only resolution 1 gives rise to a molecule with a distal exchange and without a long heteroduplex. In the second alternative, migration of a double Holliday junction and resolution can lead to a CO with a distal exchange point and with small stretches of heteroduplex DNA.

ates with a long heteroduplex on one side of the DSB or through migration of the double Holliday junction. Interestingly, long heteroduplex intermediates lead to a distal exchange product without (or with short) heteroduplex only for one type of resolution (orientation 1 in Figure 5). The short heteroduplex formed could be either undetectable or repaired by a short patch repair pathway. Resolution in orientation 2 generates a product with a long heteroduplex and a reciprocal product with exchange points close to initiation. The detection of an increased number of events extending to more distal positions has been observed in *pms1* and *msh2* mutants of *S. cerevisiae* (ALANI *et al.* 1994). Such observations might indicate either that the heteroduplex extends to distal positions even in a MutL wild-type context or that the absence of mismatch repair leads to an increase in heteroduplex length. Furthermore, the detection of molecules with mosaic structures suggests that heteroduplex DNA has been repaired in a non-continuous way, possibly by an alternative repair pathway, such as the short patch repair pathways described in *msh2* and *pms2* mutant strains of *S. cerevisiae* and *S. pombe* (FLECK *et al.* 1999; COIC *et al.* 2000; HOFFMANN *et al.* 2005) and in *msh6* mutants of *Drosophila melanogaster* (RADFORD *et al.* 2007b).

In conclusion, our work provides precise information on the activity of MLH3 and its major partner, MLH1.

The activity of MLH3 on crossover control does not seem to be redundant to that of PMS2 or PMS1 in mice, and this meiosis-specific property of MLH3 seems to be conserved among species. In *Arabidopsis thaliana* *Mlh3* mutants show a defect in chiasma formation with 30% residual activity (FRANKLIN *et al.* 2006). In some species, such as *Caenorhabditis elegans*, *D. melanogaster*, and *S. pombe*, *Mlh3* is absent and it is postulated that either *Pms2* substitutes for the CO function of *Mlh3* in these species or crossover control does not require the MutL γ complex. This hypothesis could be consistent in *D. melanogaster* and *S. pombe*, where the nature of recombination intermediates might differ from those occurring in mice as suggested by the requirement for COs on *Mus81* in *S. pombe* (SMITH *et al.* 2003) and on *mei9* in *D. melanogaster* (RADFORD *et al.* 2007a). The crossover control activity of this MutL γ complex is thought to require the MutS γ complex (MSH4 and MSH5) on the basis of immunolocalization and interaction analysis. Functional analyses in both *S. cerevisiae* and *C. elegans* have shown that *Msh4* was indeed required for crossover formation. In mice, however, testing directly the effect of MSH4 or MSH5 on crossing over is not currently feasible given that these mutants have earlier defects in the process of recombination, disrupting the progression of meiotic prophase probably before DSBs can eventually be repaired. In light of the current re-

sults, together with previous studies demonstrating the importance of MLH1 in the majority of CO events in mice, we hypothesize that MSH4–MSH5 heterodimers function at the same cohort of CO events, suggesting that the residual 5–10% of COs that are not dependent on mismatch repair function are instead processed through an alternative pathway that is analogous, functionally if not compositionally, to the yeast Mus81 pathway.

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