

The DNA mismatch-repair MLH3 protein interacts with MSH4 in meiotic cells, supporting a role for this MutL homolog in mammalian meiotic recombination

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Received February 13, 2002; Revised and Accepted May 15, 2002

DDBJ/EMBL/GenBank accession no. AY090776

The mismatch-repair (MMR) system plays a central role in maintaining genetic stability and requires evolutionarily conserved protein factors, including MutS and MutL homologs. Since the discovery of a link between the malfunction of post-replicative MMR and human cancers, a number of works have focused on the function of MutS and MutL homologs in the correction of replication errors. However, several MutS-like and MutL-like proteins also participate in meiotic recombination. The MutL homolog MLH3 has been recently identified in mammals. Several pieces of evidence support a role for this protein in post-replicative MMR. To investigate whether MLH3 also acts during meiotic recombination, we analyzed its expression in mammalian germ cells. The *MLH3* gene is expressed in mouse meiotic cells and in human testis, and, as revealed by immunoprecipitation assays, the MLH3 protein is found in mouse spermatocytes. We further demonstrate that the meiosis-specific MSH4 protein, known to participate to meiotic recombination, is co-immunoprecipitated with MLH3 from mouse meiotic cell extracts. In addition, the two MLH3 protein isoforms potentially expressed in human testis (hMLH3 and hMLH3 Δ 7) interact *in vitro* with the hMSH4 protein. These interaction data suggest that MLH3 is associated with MSH4 in mammalian meiotic cells, and strongly support the possibility that MLH3 plays a role in mammalian meiotic recombination.

INTRODUCTION

The MutS and MutL proteins are critical components of the post-replicative DNA mismatch-repair (MMR) system in *Escherichia coli* (1,2). Although the MMR system is highly conserved, the situation in eukaryotes is more complex, since several MutS homologs (MSH) and MutL homologs (designated either MLH or PMS) have been identified. Among all the MutS- and MutL-like human proteins, hMSH2, hMSH3, hMSH6, hMLH1 and hPMS2 are involved in the correction of replication errors (1,3,4). Mismatch recognition requires available hMutS- α (hMSH2–hMSH6) or hMutS- β (hMSH2–hMSH3) heterodimeric complexes. hMutS- α recognizes base–base mismatches and insertion–deletion loops (IDLs), whereas hMutS- β primarily mediates the repair of IDLs. An hMLH1–hPMS2 heterodimer is also implicated in MMR. During the repair process, the hMLH1–hPMS2 complex most probably combines with either hMutS- α or hMutS- β and the DNA mismatch to form a ternary complex. In human cells, a second MutL heterodimeric complex (hMLH1–hPMS1) has been identified, but its involvement in MMR remains to be demonstrated (5). The role of hMutL homologs in reparation process is not clearly understood.

However, it has been speculated that these proteins coordinate the interplay between the mismatch-recognition complex and other proteins necessary for MMR (1).

Deficiencies in MMR are associated with inherited and sporadic cancers (6). These cancers display microsatellite instability (MSI). To date, germline mutations in MMR genes have been found in 60–70% of hereditary non-polyposis colorectal cancers (HNPCC). Alterations of *hMSH2* and *hMLH1* are responsible for most HNPCC familial cases in which the underlying mutation has been identified, while mutations in *hMSH6* and *hMPS2* are less frequent. Alteration of *hPMS1* has been reported in only one patient. Because the causative mutation is found only in a subset of HNPCC families, it has been speculated that additional genes underlying MSI-positive familial colorectal cancers remain to be discovered.

Lipkin and colleagues (7) have cloned the mammalian homolog of the *Saccharomyces cerevisiae* DNA MMR gene *MLH3*. It has been proposed that loss of hMLH3 function might be associated with colorectal cancer susceptibility, based on the following evidence. First, in mammals as in yeast, MLH3 interacts with MLH1. Second, overexpression of a dominant-negative MLH3 mutant induces microsatellite

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instability in mammalian cells. Third, the mouse *MLH3* gene lies within the colon cancer susceptibility locus *Ccs1* (7). In order to determine whether *hMLH3* inactivation is involved in colorectal cancers, screening of both germline and somatic *hMLH3* mutations in patients with MSI-positive colorectal carcinomas has been performed. To date, three studies have lead to rather conflicting conclusions (8–10). Although further analyses are required to establish the actual frequency and pathogenic role of *hMLH3* inactivation in different tumor types, it seems that germline *hMLH3* mutations are not frequently involved in HNPCC families. This finding is consistent with the effect of loss-of-function mutations in yeast, which indicates a minor role for the Mlh3 yeast protein (Mlh3p) in MMR (11,12).

In contrast, Mlh3p plays an important role in meiotic recombination (12). In *mlh3* yeast mutants, meiotic crossover is reduced to approximately 70% of the wild-type level. Since crossovers are essential for the proper segregation of homologous chromosomes at the first meiotic division, *mlh3* yeast mutants exhibit failure in chromosome disjunction and consequently a diminution of spore viability.

Involvement of MutS and MutL homologs during meiosis has already been reported. They act at at least two levels: in repairing mismatches in heteroduplex DNA formed during the process of meiotic recombination and, most probably, in promoting crossover. In yeast, mutations in *Mlh1* reduce the level of crossovers (12,13). Functional studies strongly suggest that Mlh1p and Mlh3p act as a heterodimeric complex to promote meiotic crossover (12). On the basis of genetic analyses, the Mlh1p–Mlh3p heterocomplex appears to function in conjunction with Msh4p and Msh5p proteins. Msh4p and Msh5p also form a complex (14), like other MutS-like proteins. In yeast, Msh4p and Msh5p do not play any role in MMR—they are meiosis-specific and are crucial for meiotic recombination (15–17).

In mammals, *MSH4*, *MSH5* and *MLH1*, and also the MutL homolog *PMS2*, act during meiosis, as revealed by analyses of mice carrying disruptions in these genes (18–23). Further biochemical and immunolocalization studies have led to the proposal that *MSH4*, most probably associated with *MSH5*, functions notably in conjunction with *MLH1* to promote crossover (24).

To date, studies concerning *MLH3* function in mammals have focused on the possible involvement of this protein in the post-replicative MMR process. However, taking into account the *mlh3*-deficient yeast phenotype, it is tempting to speculate that, in mammals, *MLH3* is implicated in proper meiosis. Here, we report that the *MLH3* gene is expressed in mammalian meiotic germ cells. We also provide evidence that *MLH3* interacts with the meiosis-specific protein *MSH4*. These findings support the idea that, in mammals, the newly identified *MLH3* protein may be involved in meiotic recombination mechanisms.

RESULTS

Expression of the *MLH3* gene in mammalian male germ cells

Previous analyses have shown that, in mammals, the *MLH3* gene is expressed in a large number of somatic fetal and adult

tissues (7). As a first step toward investigating whether the *MLH3* protein could participate to meiotic recombination in mammals, we examined *MLH3* gene expression by RT–PCR analysis of polyadenylated RNAs isolated from mouse germ cells. To this end, we used elutriated germ cells isolated from 6-week-old mouse testes. After elutriation, these cells were isolated into several fractions according to their size. As previously described (25), the first two fractions collected contained only post-meiotic germ cells: 90% of elongated spermatids in fraction 1, and elongated and round spermatids in fraction 2 (Fig. 1A). These post-meiotic cells were also found in fraction 3 with pre-leptotene and zygotene spermatocytes. Fractions 4 and 5 contained round spermatids and zygotene spermatocytes, while 90% of cells found in fractions 6 and 7 were pachytene spermatocytes (Fig. 1A). Pachytene spermatocytes of larger size were found in fractions 8 and 9 (Fig. 1A). Fraction 10 contained multinuclear spermatocytes. Sertoli cells were not collected. In order to design specific oligonucleotides allowing amplification of the complete *mMLH3* coding sequence, we characterized the *mMLH3* cDNA 5' end (see Materials and Methods) (GenBank accession no. AY090776), and we used the *mMLH3* cDNA 3' end sequence available in the GenBank database. As shown in Figure 1B, *mMLH3* is expressed in mouse germ cells. However, no *mMLH3* messengers were found in fractions containing only post-meiotic cells (fractions 1 and 2). In contrast, *mMLH3* mRNAs were found in fractions containing meiotic cells (zygotene or pachytene spermatocytes) (Fig. 1B).

To analyse post-meiotic cell fractions, we looked at the expression of the *mPrm-1* gene (protamine 1 gene), which is transcribed in haploid cells (26) (Fig. 1B). For the purpose of comparison, we analyzed expression of the *mMSH4* and *mPMS2* genes. *mMSH4* is a MutS homolog expressed only in meiotic cells (25). *mPMS2* is a MutL homolog that is required for proper meiosis, as revealed by the phenotype of male homozygous mutant mice (23). The expression profile of *mMLH3* in testis cells was comparable to those of MutS and MutL homologs involved in mammalian meiosis (Fig. 1B). Taken together, these results suggest that the mouse *MLH3* gene is expressed in male meiotic cells.

We next checked whether the *MLH3* gene is expressed in human testis. PCR was performed on a human testis cDNA library with specific primers designed to amplify the complete *hMLH3* coding sequence. We observed that the *hMLH3* cDNA was amplified from human testis (Fig. 1C). The amplification products were cloned for further analysis. DNA sequencing revealed that the two *hMLH3* mRNAs isoforms, previously described in somatic tissues (7), are also expressed in testis. One of them contains the full-length coding sequence, while the second isoform (*hMLH3Δ7*), resulting from alternative splicing, exhibits an inframe deletion of exon 7.

Immunoprecipitation of the mouse *MLH3* protein from meiotic cell extracts

In an attempt to examine the expression of the *MLH3* protein in mammalian germ cells, we elicited rabbit antiserum against a peptide comprising amino acids 64–78 of the human and

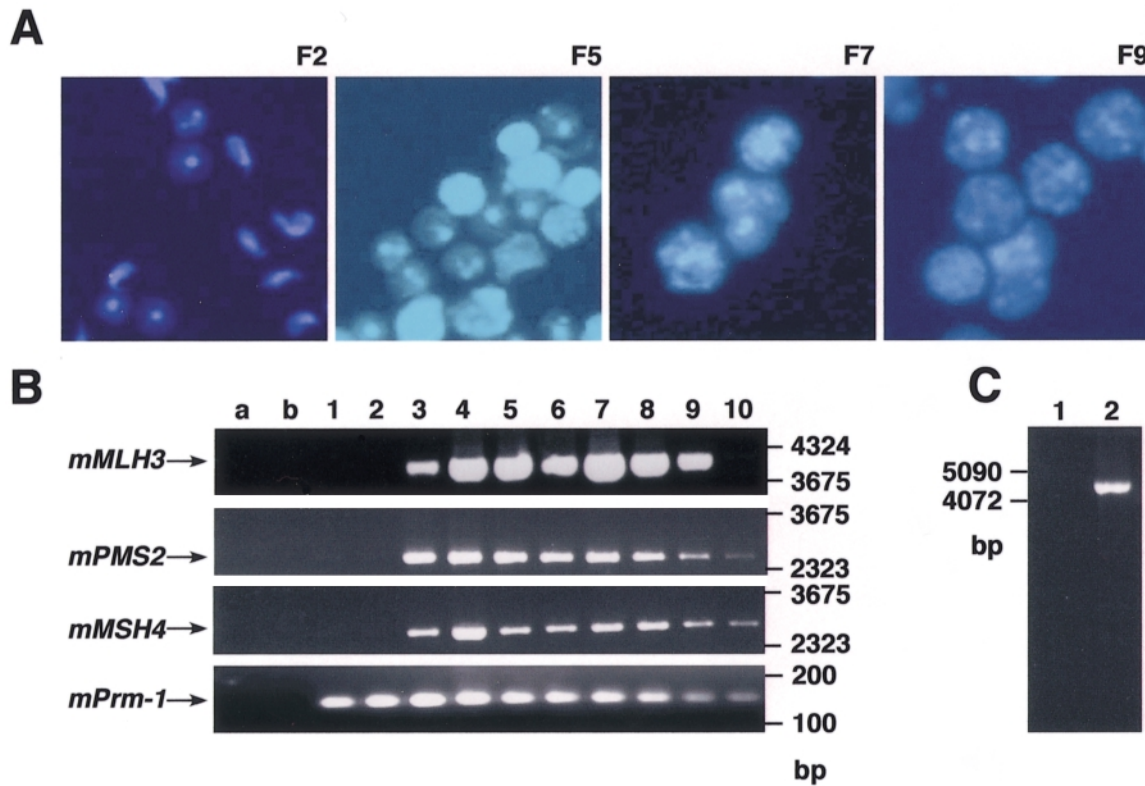


Figure 1. Expression analysis of the *MLH3* gene in mouse germ cells and human testis (A) Visualization ($\times 400$) of elutriated mouse testis cell fractions with DAPI staining. F2, elongated and round spermatids; F5, round spermatids and zygotene spermatocytes; F7, pachytene spermatocytes; F9, pachytene spermatocytes. (B) RT-PCR analysis from elutriated mouse testis cell fractions. Lane a corresponds to reactions performed in the absence of poly(A)⁺ mRNAs. Lane b corresponds to reactions carried out without the reverse transcription step. Lanes 1–10 show RT-PCR products obtained from fractions 1–10, respectively. Expression of *mMLH3* was analyzed using primers designed to amplify the nearly complete coding sequence. Expression of *mPMS2* and *mMSH4* was also analyzed. The expected sizes are, respectively, 2630 and 2764 bp. The size of the expected *mPrm-1* cDNA fragment is 122 bp. (C) Expression of the *hMLH3* gene in human testis. Lane 2: 5 μ l of a human testis cDNA library were submitted to PCR amplification using *hMLH3*-specific primers chosen to amplify the complete coding sequence (4359 bp). Lane 1 corresponds to a PCR reaction performed by omitting human testis cDNAs.

mouse MLH3 proteins. The resulting anti-MLH3 antibodies were purified by affinity chromatography. Anti-MLH3 antibodies efficiently immunoprecipitated the *in vitro* translated human MLH3 protein and did not cross-react with other *in vitro* translated proteins (Fig. 2A). These polyclonal antibodies also specifically recognize a recombinant GST-hMLH3 fusion protein in western blotting experiments (Fig. 2B). On immunohistochemistry, the MLH3 protein could not be detected in mouse tissues previously described to exhibit a high level of *mMLH3* messengers (7), suggesting that our antibodies are not functional in immunohistological assays (data not shown). Therefore, the expression of the MLH3 protein in germ cells was investigated by immunoblot analysis of whole protein extracts or by immunoprecipitation assays. These analyses were performed using extracts from elutriated mouse germ cell fractions that contained 90% of spermatocytes. When whole protein extracts from spermatocytes (10^6 cells) were analyzed directly by immunoblot, the anti-MLH3 antibodies did not allow the detection of a significant signal (data not shown). However, from extracts prepared from 50×10^6 cells, anti-MLH3 antibodies immunoprecipitated specifically a protein that migrates at approximately 160 kDa—the same position as the human MLH3 protein

(Fig. 2C). This 160 kDa mouse protein was not immunoprecipitated from post-meiotic germ cells or with pre-immune serum (Fig. 2C). These results are consistent with mRNA expression analyses, and suggest that the mouse MLH3 protein is expressed in meiotic cells.

MLH3 associates with the meiosis-specific MSH4 protein

The expression of the MLH3 protein in mammalian meiotic cells, associated with the meiotic phenotype of *mlh3*^{-/-} yeast mutants (12), led us to explore the idea that MLH3 participates in mammalian meiotic recombination. As MutL homologs are known to function in association with MutS-like proteins, we checked whether MLH3 interacts with the meiosis-specific MSH4 protein, which is known to be involved in different steps of meiotic recombination (18,24). We performed co-immunoprecipitation assays using whole extracts from mouse meiotic cells. We observed that MLH3 and MSH4 were co-immunoprecipitated by the anti-MLH3 polyclonal antibodies (Fig. 3). However, we could not co-immunoprecipitate MLH3 and MSH4 proteins using the previously characterized anti-MSH4 antibodies (24). This may be due to the fact that anti-MSH4 antibodies immunoprecipitate the

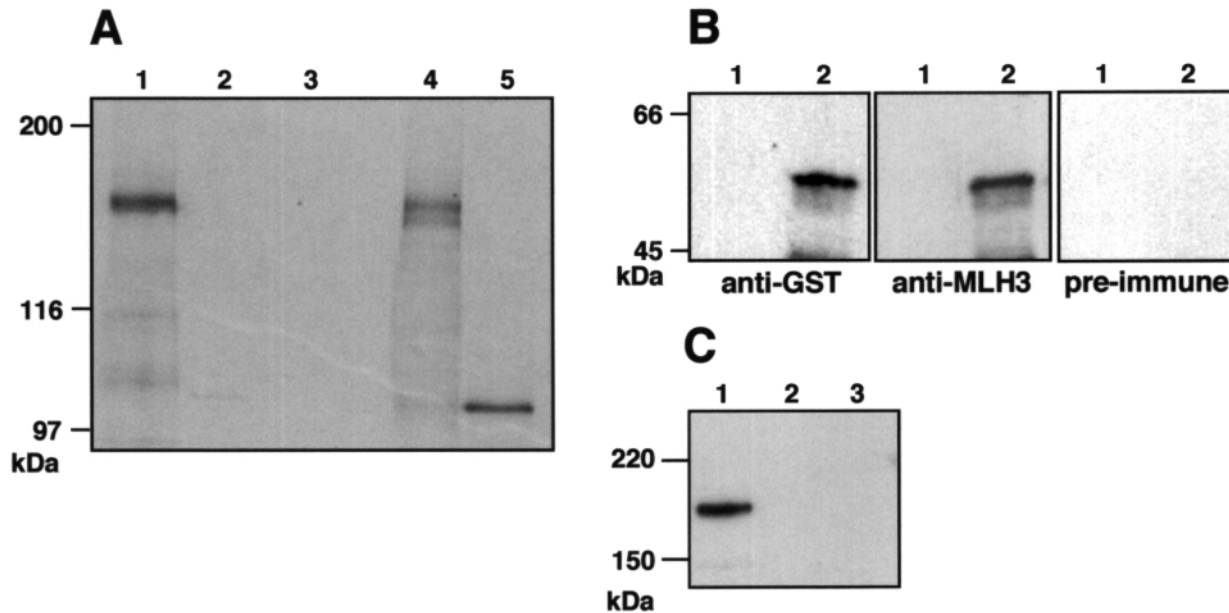


Figure 2. Anti-MLH3 polyclonal antibodies immunoprecipitate the mouse MLH3 protein from meiotic cells. (A) The human full-length MLH3 protein was translated *in vitro* and radiolabeled with [35 S]methionine. Two microliters of the *in vitro* translation reaction was subjected to immunoprecipitation by using either affinity-purified anti-MLH3 polyclonal antibodies (lane 1) or 20 μ l of the corresponding pre-immune serum (lane 2). Two microliters of the 35 S-labeled and *in vitro* translated hMSH4 protein was also submitted to immunoprecipitation with the anti-MLH3 antibodies (lane 3). Immune complexes were recovered by centrifugation, separated on an 8% polyacrylamide gel and submitted to autoradiography. Lanes 4 and 5 correspond respectively to 10% of total 35 S-labeled hMLH3 and hMSH4 used in immunoprecipitation assays. Molecular weight standards are indicated. (B) *Escherichia coli* BL21 (DE3) cells were transformed with the pGEX 4T-2-hMLH3Nt plasmid, which allows expression of the GST protein fused to the first 290 N-terminal amino residues of the hMLH3 protein. Whole-cell lysates from uninduced (lanes 1) or induced (lanes 2) bacteria were analyzed by western blotting with anti-GST antibodies, anti-MLH3 antibodies or the corresponding pre-immune serum. Both anti-GST and anti-MLH3 antibodies allow the detection of a protein of the expected size (60 kDa) in induced bacterial cells. (C) Anti-MLH3 antibodies were used to perform immunoprecipitation assays from elutriated mouse testis cell fractions containing either spermatocytes (fractions 6–9, lane 1) or post-meiotic cells only (fractions 1 and 2, lane 3). As a negative control, lysates from mouse spermatocytes were also submitted to immunoprecipitation with the pre-immune serum (lane 2). Lysates from 5×10^7 cells were used in each immunoprecipitation assay. The resulting immunoprecipitates were analyzed by western blotting using the anti-MLH3 antibodies. A protein that migrates at a 160 kDa position was specifically immunoprecipitated from mouse spermatocytes using affinity-purified antibodies raised against MLH3.

MSH4 protein with a very low efficiency, as indicated by immunoprecipitation assays performed with the *in vitro* translated MSH4 protein (data not shown). Alternatively, this failure may result from disruption of the protein complex by the anti-MSH4 antibodies. These results led us to further investigate the association between MLH3 and MSH4. To this end, we performed GST pull-down assays using the *in vitro* translated and radiolabeled human MLH3 protein and the GST-hMSH4 protein bound to Sepharose beads. GST and GST-RhoA were used as negative controls and GST-hMLH1 provided a positive control, since it has been demonstrated that the hMLH1 protein interacts with hMLH3 (7). We observed that radiolabeled hMLH3 protein was pulled down by GST-hMSH4 protein and, as expected, by GST-hMLH1 (Fig. 4). These *in vitro* interaction data suggest that the human MLH3 protein interacts physically with the meiosis-specific hMSH4 protein. They are consistent with *in vivo* interaction results, and suggest that co-immunoprecipitation of murine MLH3 and MSH4 proteins may be due to a direct protein-protein association in meiotic cells.

Two *MLH3* mRNAs isoforms were found to be expressed in human testis, as in human somatic tissues. These observations raised the question of the functional specificity of the two corresponding proteins. The *hMLH3 Δ 7* isoform is predicted to

encode a protein lacking an amphipathic helix with high amino acid identity to bacterial, yeast and mammalian homologs. Using mammalian two-hybrid assays, Lipkin and colleagues (7) found that, in contrast to the hMLH3 full-length protein, hMLH3 Δ 7 failed to interact with hMLH1, suggesting that the conserved C-terminal helix encoded by *hMLH3* exon 7 is critical for the association with hMLH1. In contrast, Kondo and colleagues (27) reported that hMLH3 Δ 7 has a strong interaction with hMLH1 in yeast two-hybrid and GST pull-down assays. Consequently, it remains unclear whether hMLH3 and hMLH3 Δ 7 differ in their ability to bind hMLH1. As these two proteins are potentially expressed in human testis, we asked whether they could both interact with hMSH4. Therefore, we performed GST pull-down assays using the *in vitro* translated hMLH3 Δ 7 protein. In agreement with Kondo and colleagues, we observed that hMLH3 Δ 7 was efficiently pulled down by GST-hMLH1. Similarly, this protein was precipitated by GST-hMSH4. These findings suggest that the two hMLH3 isoforms potentially expressed in human testis could interact with hMSH4, and they further indicate that the C-terminal region encoded by *hMLH3* exon 7 is not crucial for this interaction. Both *in vivo* and *in vitro* interaction data led us to suggest that MLH3 and MSH4 proteins are part of a same protein complex in mammalian meiotic cells.

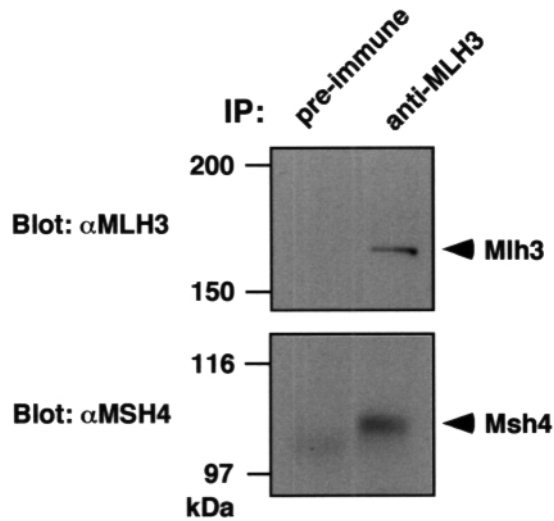


Figure 3. Co-immunoprecipitation of MLH3 and MSH4. Lysates from elutriated testis cell fractions 6–9 (5×10^7 cells) were subjected to immunoprecipitation with either 2 μ g anti-MLH3 antibodies or pre-immune serum, as indicated. Immunoprecipitates were analyzed by western blotting using anti-MLH3 (top) and anti-MSH4 antibodies (bottom). The 104 kDa Msh4 protein was co-immunoprecipitated with the Mlh3 protein.

DISCUSSION

In both yeast and mammalian cells, members of the DNA MMR family are involved in the correction of replication errors. In addition, these MMR factors appear to perform several functions during meiotic recombination: repair of mismatches in heteroduplex DNA, inhibition of recombination between diverged sequences and, most probably, resolution of recombination intermediates. The mammalian MutS homologs MSH2, MSH3 and MSH6 are crucial for post-replicative MMR—but these proteins do not appear to play an essential role during meiosis, since no meiotic phenotype is apparent in *Msh2*^{-/-}, *Msh6*^{-/-} or *Msh3*^{-/-} mice. In contrast, the MSH4 and MSH5 proteins do not participate in post-replicative repair, but are required for proper meiosis, although the precise functions of these two MutS homologs are not fully understood. The situation concerning MutL homologs is much more complex. Indeed, mouse models carrying loss-of-function alleles of the *MLH1* and *PMS2* genes have established that these MutL-like proteins are involved both in post-replicative mismatch repair and in meiotic recombination. First, these mutant mice display a predisposition to tumors (21,22,28,29). Second, *Pms2*-deficient males and both *Mlh1*^{-/-} males and females are infertile (21–23). Male *Pms2*^{-/-} mutants produce only abnormal spermatozoa. About 80% of *Pms2*-deficient spermatocytes show abnormalities in meiotic prophase I characterized by anomalies in chromosome synapsis. Male mice with mutations in the *MLH1* gene do not produce spermatozoa and, in females, ovulations are rare. Chromosome synapsis occurs normally in spermatocytes and oocytes of MLH1 null mice. However, cytological analyses revealed that sterility of these mutant mice results from drastically reduced meiotic recombination. These findings indicate that these two murine MutL homologs are implicated in proper meiosis,

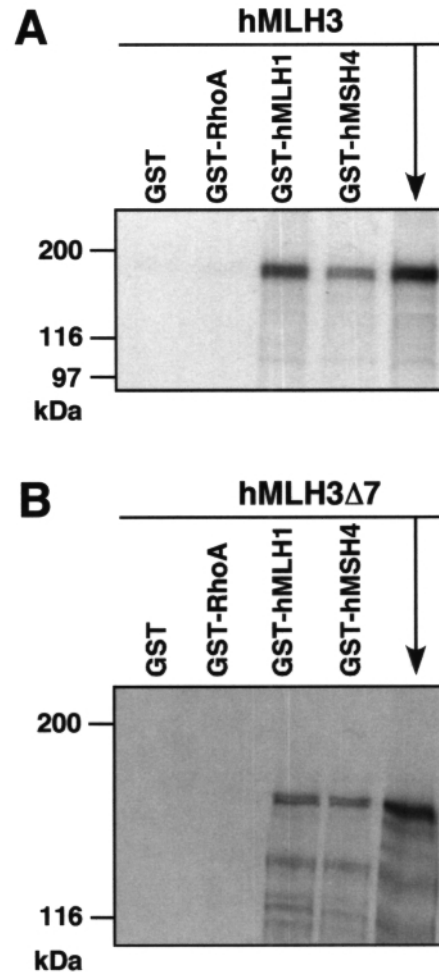


Figure 4. The hMSH4 protein interacts with the two human MLH3 protein isoforms in GST pull-down assays. (A) *In vitro* interaction between hMSH4 and hMLH3. Two microliters of ³⁵S-labeled hMLH3 protein were added to glutathione S-Sepharose beads coated with GST, GST-RhoA, GST-hMLH1 or GST-hMSH4, as indicated. Materials retained on beads were separated by electrophoresis on a 7% denaturing polyacrylamide gel, and labeled proteins were detected by autoradiography. Ten percent of the total *in vitro* translation mixture used in each assay (0.2 μ l of ³⁵S-labeled hMLH3 protein) was also submitted to the same analysis as input material control (last lane). hMLH3 protein was efficiently precipitated with both GST-hMLH1 used as a positive control and GST-hMSH4. (B) *In vitro* interaction of hMSH4 and hMLH3 Δ 7. GST pull-downs were also performed using 20 μ l of ³⁵S-labeled hMLH3 Δ 7 protein with the indicated GST fusion proteins. Ten percent of the total *in vitro* translation mixture used in each assay (0.2 μ l of ³⁵S-labeled hMLH3 Δ 7 protein) was also analyzed (last lane).

although they act at different stages: PMS2 is involved in synapsis of homologous chromosomes early in meiotic prophase, while MLH1 acts later in prophase and appears to be required for crossing-over formation, as described in yeast (13). Lipkin and colleagues (7) have identified another MutL homolog gene, *hMLH3*. A question arising from this finding is whether the MLH3 protein, in mammals, is involved both in post-replicative MMR and in meiotic recombination, as described for PMS2 and MLH1.

The study presented here was carried out to investigate the possible involvement of MLH3 in mammalian meiotic

recombination. Our results establish that the murine *MLH3* gene is expressed in male meiotic cells. In agreement with this finding, we observed that the human *MLH3* cDNA was amplified from a testis cDNA library.

To investigate the expression of the mammalian MLH3 protein in meiotic cells, anti-MLH3 rabbit polyclonal antibodies, raised against a peptide found in the N-terminal region of both human and mouse proteins, were generated. Although these antibodies did not function in immunohistological assays, they immunoprecipitated a 160 kDa protein from mouse spermatocytes. In contrast, this protein was not detected in post-meiotic mouse germ cells. Based on the molecular weight of the human MLH3 protein (160 kDa) and on the size of the mouse *MLH3* coding sequence (Fig. 1B), 160 kDa is the expected size of the mouse MLH3 protein. Furthermore, since the immunoprecipitation data are consistent with mRNA expression analyses, we concluded that the protein detected specifically by our antibodies corresponds to the mouse MLH3 protein, which appears to be expressed in mouse spermatocytes. Based on mouse mRNAs and protein expression data, it appears likely that the MLH3 protein is expressed in human spermatocytes, although further studies are required to definitively establish this point. Whether MLH3 protein is expressed in mouse and human oocytes remains to be determined. Nevertheless, the expression data reported here are consistent with a possible involvement of MLH3 in mammalian meiosis.

To explore this possibility further, we asked whether MLH3 interacts with proteins involved in meiotic recombination. Genetic and biochemical analyses in yeast and mammals establish that heterodimeric complexes of MutS homologs interact with heterodimers of MutL-like proteins during the MMR process. Nevertheless, the exact nature of the protein-protein contacts that govern the assembly of these MMR protein complexes is unknown. Purified yeast Msh2p has been shown to interact with the Mlh1p-Pms1p heterodimer (30), suggesting that this MutS homolog, which is a mutual component of all MutS mitotic heterocomplexes, may play a central role in the formation of the repair protein complexes. During the meiotic recombination process, the yeast and mammalian MSH4 and MSH5 proteins also appear to function as a heterodimer (14,31–33). Likewise, the yeast Mlh1p and Mlh3p proteins act as a heterocomplex to promote meiotic crossovers (12). Furthermore, several studies indicate that a physical and functional link exists between MutS and MutL homologs involved in meiotic recombination, as is the case in the MMR process. First, genetic analyses in *Saccharomyces cerevisiae* suggest that Msh4p-Msh5p and Mlh1p-Mlh3p heterodimers function in a common pathway crucial for crossover formation (12,13,15,16). Second, biochemical studies in mammals have shown that MSH4 interacts physically with MLH1 and that these two proteins co-localize on mice meiotic chromosomes (24). These observations prompted us to investigate whether the mammalian MLH3 protein interacts with the meiosis-specific MutS homolog MSH4. We observed that MLH3 and MSH4 could be co-immunoprecipitated from mouse spermatocyte extracts by using anti-MLH3 antibodies. Furthermore the human MLH3 and MSH4 proteins appeared to physically interact in *in vitro* GST pull-down assays. These results suggest that these two proteins belong to the

same complex in mammalian male meiotic cells. Since the mammalian MLH3 and MSH4 proteins can both associate with MLH1, one can assume that the MLH3/MSH4 interaction detected in immunoprecipitation assays is mediated by the MLH1 protein. However, GST pull-down results rather suggest that co-immunoprecipitation of both proteins may be due to a direct interaction. Whether several MLH3 isoforms are expressed in mouse meiotic cells remains to be determined. However, our data suggest that two MLH3 protein isoforms (hMLH3 and hMLH3 Δ 7) are potentially expressed in human testis. *In vitro* interaction experiments revealed that these two isoforms do not differ in their ability to bind MSH4, and they further indicate that the highly conserved C-terminal region lacking in the hMLH3 Δ 7 isoform is not crucial for association with MSH4.

The interaction data reported here strongly support the idea that, in mammals, MLH3 is involved in meiotic recombination events and functions in conjunction with MSH4. The MSH4 protein appears to participate to different steps of mammalian meiotic prophase. First, chromosome synapsis in mice is more dependent on the MSH4-MSH5 complex than is synapsis in yeast (17–20). Indeed, *MSH4* and *MSH5* homozygous mutant mice show a severe defect of synapsis, indicating that an MSH4-MSH5 complex plays an important role as soon as synapsis occurs (18–20). Second, immunolocalization analyses on mice meiotic chromosomes have shown that MSH4 is associated with chromosomes not only at the step of synapsis but also later, at the time when crossovers occur (24). During this stage of meiotic prophase, MSH4 co-localizes with MLH1 at sites of crossover. These findings, associated with the meiotic phenotype of the *Mlh1*^{-/-} mice, indicate that MSH4, most probably associated with MSH5, functions later in conjunction with MLH1 to promote crossover. Analyses of mice carrying a disruption of the *MLH3* gene, associated with immunolocalization studies, would be useful to determine whether MLH3 acts at the step of chromosome synapsis and/or at the step of crossover formation.

In mammals, the interplay between MMR factors and the several steps of the mammalian meiotic recombination process is not clearly understood. These proteins may participate in different mechanisms essential for proper meiosis. In *Saccharomyces cerevisiae*, the Msh4p and Msh5p proteins are not required for meiotic MMR and gene conversion (15,16). However, we cannot exclude the possibility that MSH4 and MSH5 participate in the reparation of mismatches in heteroduplex DNA during mammalian meiosis. Likewise, one can assume that MLH3 participates in meiotic mismatch correction in mammals. Another attractive possibility is that these MMR factors act to prevent meiotic recombination between diverged sequences. In addition, several features suggest that MLH1, MLH3, MSH4 and MSH5 form a complex that promotes formation of crossovers at late meiotic prophase in mammals, as is the case in yeast (12). Biochemical analyses are necessary to test these several possibilities, which are not mutually exclusive. Likewise, further investigations are required to understand the functional relationship between MutS and MutL homologs involved in mammalian meiosis. The results presented here provide essential information for these further studies, since they suggest that MLH3 is one of the MMR factors involved in the mammalian meiotic recombination process.

MATERIALS AND METHODS

All the oligonucleotides used in this study are presented in Table 1.

Cloning of the mouse *MLH3* cDNA 5' end

A BLAST homology search was performed with the human *MLH3* cDNA sequence against the expressed sequence tag (EST) database. An EST derived from a 16-day-old mouse heart (GenBank accession no. BB518976) was identified. It encodes an amino acid sequence showing 87% identity and 91% similarity with the region located between amino residues 134 and 347 of the human *MLH3* protein. On the basis of this partial cDNA sequence, *mEST-1* and *mEST-2* oligonucleotides were designed in order to perform rapid amplification of the 5' cDNA end (5'-RACE) using mouse testis Marathon-Ready cDNA (Clontech). A first amplification round was carried out using the primer *mEST-1* and the adaptor primer AP1 (Clontech). Amplification products were used in a second PCR reaction performed with the nested specific primer *mEST-2* and the nested adaptor primer AP2 (Clontech). A 1 kb PCR product was obtained and cloned into the pCR3.1 vector using the TA-cloning kit (Invitrogen). Positive clones were characterized by DNA sequencing. They were found to contain a 1068 bp cDNA fragment that contains an open reading frame (GenBank accession no. AY090776) encoding a polypeptide sequence (337 amino acid residues) sharing 86% identity and 91% similarity with the first 337 amino acids of the human *MLH3* protein. Furthermore, the polypeptide sequence deduced from the cloned cDNA was identical to the previously reported sequence corresponding to the first 270 amino acid residues of the mouse *MLH3* protein (7). The cloned cDNA fragment also contains 54 nucleotides upstream of the translational start codon.

Isolation of mouse spermatocytes fractions

Investigations were conducted in accordance with institutional guiding principles for the care and use of animals. Forty testes of 6-week-old mice were submitted to centrifugal elutriation as previously described (25). Testicular cells were separated in 10 fractions according to their size. The composition of each fraction was analyzed by DAPI staining.

Expression analysis

To analyze the expression of several genes in mouse germ cells, polyadenylated mRNAs were prepared from elutriated cell fractions using the mRNA Isolation Kit (Roche Molecular Biochemicals) according to the supplier's instructions. One microgram of mRNAs was used as template for a reverse-transcriptase reaction using Expand Reverse Transcriptase (Roche Molecular Biochemicals) and an oligo(dT) primer. Five microliters of the cDNA synthesis reaction were used for subsequent PCR amplifications. *mMSH4* and *mPMS2* cDNAs were amplified using standard PCR conditions with specific primers allowing the amplification of the complete coding regions: *mMSH4*-forward, *mMSH4*-reverse and *mPMS2*-forward, *mPMS2*-reverse, respectively. Using the same procedure, a *mPrm-1* (mouse protamine 1) cDNA fragment was amplified with primers designed to perform amplification across the junction between the two exons of this gene: *mPrm-1*-forward and *mPrm-1*-reverse. The sequences of the *mMLH3* cDNA 5' region (GenBank accession no. AY090776) and the *mMLH3* cDNA 3' end (GenBank accession no. BC003865) were used to choose primers allowing amplification of the *mMLH3* cDNA (the whole coding sequence lacking the first 181 and last 24 nucleotides). A first PCR was performed using the primers *mMLH3*-forward and *mMLH3*-reverse.

Table 1. Primers used in this study

Oligonucleotide	Sequence ^a
<i>mMsh4</i> -forward	5'-GGGCAGGTAGATGCTGAGG-3'
<i>mMsh4</i> -reverse	5'-CCTGTGCACACCGTGTACTG-3'
<i>mPms2</i> -forward	5'-ACTGTGACTGGAGGAGTCCTGCAT-3'
<i>mPms2</i> -reverse	5'-ACTCTATGCTACAAGGGGTGTGTC-3'
<i>mPrm-1</i> -forward	5'-GCCAGATACCGATGCTGC-3'
<i>mPrm-1</i> -reverse	5'-CTTATGGTGTATGAGCGGC-3'
<i>mEST-1</i>	5'-TTACTCCTTCCTGAATACAAATCAAC-3'
<i>mEST-2</i>	5'-CAAATCAACACGGTATCCCAGC-3'
<i>mMlh3</i> -forward	5'-CCTTGGGCCAGTGTGTTGAAGAACT-3'
<i>mMlh3</i> -reverse	5'-ATGGAGGCTACAAGGACGTATAG-3'
<i>mMlh3a</i>	5'-TGGCGGGGACGATGTAGAGAA-3'
<i>mMlh3b</i>	5'-GCTGCTGCAGGTTCTGCTCTGTTT-3'
<i>hMLH3</i> -forward	5'-CTCACCATCTTTCTACCTACC-3'
<i>hMLH3</i> -reverse	5'-TAGACCAGTGATTCTGTTC-3'
<i>hMLH3a</i>	5'-GGAATTCATATGATCAAGTGCTTGTTCAGT-3'
<i>hMLH3b</i>	5'-TCCCCCGGGTTCATGGTGGCTCACAGGGA-3'
<i>hMLH3c</i>	5'-CGGGATCCCGCATGATCAAGTGCTTGTTCAGTTGA-3'
<i>hMLH3d</i>	5'-CGGAATTCATTTGCTACTGGTGGGACCATT-3'

^aRestriction sites used in subcloning are italicized.

The subsequent amplification reaction was carried out with the nested primers *mMLH3a* and *mMLH3b*.

To analyze the expression of the *MLH3* gene in human testis, a two-step PCR procedure, using a human testis Marathon-Ready cDNA library (Clontech) as template, was performed. Oligonucleotides were designed to amplify the whole *hMLH3* coding sequence. The first PCR reaction was carried out using the *hMLH3*-forward and *hMLH3*-reverse primers. The resulting amplification products (0.5 μ l) were used as template for an additional 25 cycles of PCR performed with the nested oligonucleotides *hMLH3a* and *hMLH3b*, which contain *Nde*I and *Sma*I restriction sites, respectively. The amplification products were analyzed by electrophoresis and DNA sequencing.

Plasmids

The *hMLH3* cDNA obtained by PCR amplification was cloned directly into the pCR3.1 vector. After bacterial transformation, individual clones were sequenced. In addition to the clones containing the complete *hMLH3* cDNA, we obtained clones with an alternatively spliced product of *hMLH3* cDNA. The resulting plasmids were designated pCR3.1-hMLH3 and pCR3.1-hMLH3 Δ 7, and were used for *in vitro* translation of the hMLH3 and hMLH3 Δ 7 proteins, respectively. The pGEX 4T-2-hMLH3Nt plasmid allows expression of the GST protein fused to the first 290 amino acid residues of the hMLH3 protein. This vector was generated as follows. The cDNA insert was amplified from a human testis Marathon-Ready cDNA library using, in a first PCR reaction, the primers *hMLH3*-forward and *hMLH3*-reverse. A second amplification reaction was performed with the primers *hMLH3c* and *hMLH3d* containing a *Bam*HI and an *Eco*RI restriction site, respectively. Amplification products were digested with both *Bam*HI and *Eco*RI and ligated between the corresponding sites of the pGEX 4T-2 vector (Novagen). To produce hMSH4 and hMLH1 as GST fusion proteins, full-length *hMSH4* and *hMLH1* coding sequences were introduced into the pGEX 5X-1 vector (Novagen). For *in vitro* production of the hMSH4 protein, the *hMSH4* cDNA was cloned into the pCR3-1 vector. All the cDNA inserts obtained by PCR amplification were confirmed to be free of mutations by DNA sequencing. The pGEX 2T-RhoA was a gift from A. Hall; this plasmid allows expression of the RhoA protein with an N-terminal GST tag (34).

Antibodies

The rabbit polyclonal anti-MSH4 antibodies recognize both human and mouse MSH4 proteins. These antibodies have been described previously (24) and used in immunoprecipitation, western blotting and immunolocalization experiments (18,24,35). Anti-MLH3 polyclonal antibodies were generated by injection into rabbits of the peptide DDVEKVGNYFTSKC conjugated to KLH. This peptide is found in the N-terminal region (amino acids 64–78) of both human and mouse MLH3 proteins. The antiserum was affinity-purified against the synthetic peptide coupled to EAH-Sepharose (Eurogentec). The horseradish peroxidase-conjugated anti-rabbit antibodies were from Amersham Biosciences.

Immunoprecipitations

Immunoprecipitations with *in vitro* translated proteins. Two microliters of the ³⁵S-labeled hMLH3 or hMSH4 proteins, diluted in 500 μ l of 20 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 750 μ g/ml bovine serum albumin (BSA), 5 mM EDTA and 5 mM dithiothreitol (DTT) with complete protease inhibitor cocktail (Roche Molecular Biochemicals), were incubated with either 2 μ g of affinity-purified anti-MLH3 antibodies or 20 μ l of the corresponding pre-immune serum for 1 h at 4°C. Fifty microliters of a 50% slurry of protein A-Sepharose beads (Amersham Biosciences), pre-equilibrated in the dilution buffer, were added for 1 h at 4°C with gentle agitation. The beads were recovered by centrifugation, washed five times in dilution buffer and boiled with 30 μ l of sodium dodecyl sulfate (SDS) sample buffer. Immune complexes were separated by electrophoresis on reducing 7% polyacrylamide-SDS gel and analyzed by autoradiography.

Immunoprecipitations from mouse germ cells. Fractions of mouse elutriated germ cells containing spermatocytes (fractions 6–9) or post-meiotic germ cells (fractions 1 and 2) were pooled. These germ cells were then resuspended to 4 \times 10⁷ cells/ml in lysis buffer: 10 mM Tris-HCl pH 8, 50 mM NaCl, 1% NP40 and, 2 mM DTT with complete protease inhibitor cocktail. Subsequently, cells were disrupted in a Dounce homogenizer, and, after incubation on ice for 30 min, the lysate was treated with DNase I (300 u/ml) for 30 min at 25°C and submitted to centrifugation at 14 000 *g* for 20 min at 4°C. The resulting supernatants were incubated for 1 h at 4°C with either 2 μ g of affinity-purified rabbit anti-MLH3 or 20 μ l of the corresponding pre-immune serum. Fifty microliters of a 50% slurry of protein A-Sepharose beads, pre-equilibrated in lysis buffer containing 2% of BSA, were added for 1 h at 4°C with gentle agitation. The beads were recovered by centrifugation, washed in lysis buffer and boiled with 30 μ l of SDS sample buffer. The released immune complexes were separated on reducing 7% polyacrylamide-SDS gel and analyzed by western blotting. Affinity-purified rabbit anti-MLH3 (1 : 1000) or rabbit polyclonal anti-MSH4 (1 : 2000) were used as primary antibodies. The secondary antibodies were goat anti-rabbit antibodies conjugated with horseradish peroxidase (1 : 10 000). Detection was performed using the ECL system (Amersham Biosciences).

GST fusion protein interaction assays with *in vitro* translated products

The production of GST fusion proteins in *E. coli* BL21 (DE3) cells has been reported previously (24). The ³⁵S-labeled hMLH3 and hMLH3 Δ 7 proteins were generated from pCR3.1-hMLH3 and pCR3.1-hMLH3 Δ 7 plasmids by *in vitro* translation using the TNT T7 coupled transcription-translation system (Promega) and [³⁵S]L-methionine (Amersham Biosciences). Induced bacterial cells that express the various GST fusion proteins were used to prepare lysates as follows. The pellet was resuspended in buffer A: 20 mM Tris-HCl, pH 8, 500 mM NaCl, 1% Nonidet P-40, 10% glycerol and 5 mM DTT, with complete protease inhibitor cocktail (1 volume of buffer per 10 volumes of bacterial culture). A 30 min digestion on ice

with 1 mg/ml lysozyme followed. After sonication, lysates were incubated with DNase I (300 u/ml) for 30 min at 25°C and centrifuged at 10 000 g for 30 min at 4°C. Glutathione S-Sepharose 4B beads (Amersham Biosciences), pre-equilibrated in buffer A plus 2% BSA, were added to lysate, such that about 200 ng of GST fusion proteins were bound to 50 µl of beads. After incubation for 1 h at 4°C under gentle agitation, the coated GST fusion protein beads were washed in buffer B: 20 mM Tris-HCl pH 8, 150 mM NaCl, 1% Nonidet P-40, 10% glycerol, 750 µg/ml BSA, 5 mM EDTA and 5 mM DTT with complete protease inhibitor cocktail. The binding reaction was performed in 1 ml of buffer B with 50 µl of coated GST fusion protein beads and different volumes of *in vitro* translated products (either 2 µl of IVTT-hMLH3 or 20 µl of IVTT-hMLH3Δ7), such that equal amount of radiolabeled proteins was used in each assay. After 1 h of incubation at 4°C under gentle agitation, the bound complexes were sedimented by centrifugation and washed in buffer B adjusted to 500 mM NaCl. Beads were then resuspended in 50 µl of SDS-PAGE loading buffer and heated for 5 min at 95°C. Protein samples were resolved by 8% SDS-PAGE. Gels were fixed, treated with Amplify (Amersham Biosciences) and dried before exposure at -70°C. In our pull-down assays, we used, as negative controls, GST and GST-RhoA proteins. RhoA is a GTPase of the Ras superfamily that, through interaction with various effector proteins, participates in important cellular processes such as actin cytoskeleton dynamics and cell adhesion (36). Each protein-protein interaction was analyzed in four separate experiments.

ACKNOWLEDGEMENTS

We thank F. Rank for technical assistance. We are indebted to C. Gribé and R. Grattery for helping to prepare this manuscript. This work was supported by grants to V.P.-F. from the Association pour la Recherche contre le Cancer. S.S.-D. is supported by a grant from the Association pour la Recherche contre le Cancer and S.N. by a fellowship from the Ligue contre le Cancer.

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