Mutation in the Mismatch Repair Gene Msh6 Causes Cancer Susceptibility

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Summary

Mice carrying a null mutation in the mismatch repair gene Msh6 were generated by gene targeting. Cells that were homozygous for the mutation did not produce any detectable MSH6 protein, and extracts prepared from these cells were defective for repair of single nucleotide mismatches. Repair of 1, 2, and 4 nucleotide insertion/deletion mismatches was unaffected. Mice that were homozygous for the mutation had a reduced life span. The mice developed a spectrum of tumors, the most predominant of which were gastrointestinal tumors and B- as well as T-cell lymphomas. The tumors did not show any microsatellite instability. We conclude that MSH6 mutations, like those in some other members of the family of mismatch repair genes, lead to cancer susceptibility, and germline mutations in this gene may be associated with a cancer predisposition syndrome that does not show microsatellite instability.

Introduction

Mismatches in DNA may result from errors in DNA replication, genetic recombination, and chemical modification of DNA and DNA precursors. Both prokaryotic and eukaryotic systems have enzymatic systems that correct DNA mismatches. Of particular interest is the bacterial MutHLS system and its counterpart in eukaryotic cells. The bacterial system repairs single-base mismatches and small insertion/deletions in DNA (for reviews see Modrich, 1991; Kolodner et al., 1995, 1996; Modrich and Lahue, 1996). Genetic and biochemical studies in eukaryotic systems have revealed that they have a mismatch repair system similar to the bacterial MutHLS system (for reviews, see Kolodner et al., 1995, 1996; Modrich and Lahue, 1996), although more complex.

Families of genes that share homology to the bacterial mutS and are therefore referred to as mutS homologs (MSH) and mutL homologs (MLH) have been identified in yeast and mammalian cells as well as other organisms. Six MSH genes (MSH1-MSH6) and four MLH genes (MLH1-MLH3, PMS1) have been described in yeast, and many such genes have been described in mammalian cells and other organisms. Interest in the mismatch repair genes was significantly enhanced by the discovery that germline mutations in some of the mismatch repair genes leads to a genetic predisposition for colon cancer, referred to as hereditary nonpolyposis colon cancer (HNPCC, Fishel et al., 1993; Leach et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Fishel and Kolodner, 1995). HNPCC is one of the common cancer susceptibility syndromes that is inherited in an autosomal-dominant fashion. A large proportion of HNPCC family members develop colonic, endometrial, and ovarian tumors as well as tumors at other sites. The tumors are characterized by a high level of genomic instability, usually observed as changes in repeat numbers of mono- and dinucleotide repeat loci. Germline mutations in MSH2 and MLH1 are the most common among HNPCC kindreds, whereas only 1 mutation in PMS1 and 2 mutations in PMS2 have been described (Kolodner et al., 1994; Nicolaides et al., 1994; Liu et al., 1996; Peltonakmi et al., 1997).

Biochemical studies revealed that a complex of proteins recognizes and binds to mismatches in yeast and mammalian cells. These studies have shown that MSH2 is capable of forming a complex with MSH3 or MSH6 (Drummond et al., 1995; Acharya et al., 1996; Habraken et al., 1996; Marsischky et al., 1996; Palombo et al., 1996) and that each of these complexes appears to have a different mispair recognition specificity. It has also been suggested, based on yeast genetic experiments, that MSH3 and MSH6 may at least partially substitute for each other, thus accounting for the strong, general mutator phenotypes of MSH2 mutants and the distinct, partially overlapping mutator phenotypes of MSH3 and MSH6 mutants (Johnson et al., 1996; Marsischky et al., 1996; Sia et al., 1997). The genetic studies performed to date (Marsischky et al., 1996; Sia et al., 1997) support the view that the MSH2-MSH6 complex functions in the repair of single base-base mispairs and smaller insertion/deletion mispairs. In contrast, the MSH2-MSH3 complex does not function in the repair of single base-base mispairs but rather functions in the repair of insertion/deletion mispairs and is predominantly responsible...
for the repair of the larger insertion/deletion mispairs. The second complex required for mismatch repair involves MLH1 and PMS1 (PMS2 in humans) (Prolla et al., 1994; Li and Modrich, 1995).

To understand the role of the mismatch repair genes, several investigators have initiated efforts to generate mice with mutations in each of the mismatch repair genes. The results from these studies have provided novel insights into the actions of these gene products. Mice that are homozygous for mutations in Msh2 are viable (de Wind et al., 1995; Reitmair et al., 1995) but have a reduced life span (Reitmair et al., 1996), exhibit genomic instability, and have a predisposition to develop lymphomas and colonic tumors. Male mice that are homozygous for mutations in the Pms2 gene are sterile and show abnormal chromosomal synapsis during meiosis, while the females are normal (Baker et al., 1995). Mice with mutations in Mlh1, in the homozygous state, are sterile due to meiotic abnormalities (Baker et al., 1996; Edelmann et al., 1996). Both PMS2- and MLH1-deficient mice show microsatellite instability in normal tissues (Baker et al., 1995, 1996; Edelmann et al., 1996) and have predisposition to certain types of cancer (Baker et al. 1995; W. E. et al., unpublished data).

The MSH6 gene (also called GTBP and p160) was identified in mammalian cells through biochemical studies of mispair binding proteins and biochemical studies of a MSH2 complementing activity as well as through analysis of the yeast genome database. The MSH6 protein, which is 1358 aa long, was shown to be a part of the mismatch repair complex (Drummond et al., 1995), and mutations in the MSH6 gene were detected in some tumor cell lines and cell lines selected for resistance to alkylating agents (Papadopoulos et al., 1995; Risinger et al., 1996). The MSH6 mutant cell lines had weak microsatellite instability at mononucleotide repeats and little if any dinucleotide repeat instability in contrast to the strong broad spectrum repeat instability observed in Msh2, Mlh1, and Pms2 mutant cell lines (Bhattacharyya et al., 1994; Shibata et al., 1994; Boyer et al., 1995; Papadopoulos et al., 1995; Risinger et al., 1995). Because these cell lines had mutations in other genes, the precise role of Gtbp/MSH6 mutations in the onset and progression of cancer has not been determined.

To understand the role of MSH6, we cloned and characterized the mouse Msh6 gene and developed mice with a null mutation in this gene through gene targeting. In results presented here, we show that MSH6-deficient mice are viable, but homozygous mutant cells show a single nucleotide mismatch repair defect. Most interestingly, these mice have a significantly reduced life span and develop lymphomas, gastrointestinal (GI) tumors, and a number of other types of tumors in tissues including those in the liver, lung, skin, and soft tissues. Our results show that Msh6 mutation causes cancer susceptibility and suggest that mutations in this gene may be involved in hereditary cancer predisposition syndromes as well as in some sporadic tumors that do not show microsatellite instability.

Results

Isolation and Characterization of the Mouse Msh6 Gene

Palombo et al. (1995) and Papadopoulos et al. (1995) described the isolation of the human gene for the 160 kDa G-T binding protein (GTBP), which is now referred to as MSH6. The MSH6 gene is in close physical proximity to MSH2, both of which are located on human chromosome 2 within approximately 1 megabase of each other. The cloning and expression pattern of the mouse homolog was described by Corradi et al. (1996), who also localized the gene to mouse chromosome 17. We also cloned the mouse Msh6 cDNA and have obtained genomic clones for Msh6 from a mouse genomic P1 library derived from the strain 129/Ola and ascertainment

Figure 1. Structure of Mouse Msh6 Gene

(A) Schematic representation of the structure of the Msh6 gene. The boxes containing the numbers 1-10 represent the individual Msh6 exons. The size of each exon is given below each exon, and the size of each intron is given above the region between each pair of exons. These numbers were determined by sequencing each relevant region, except in the case of intron 2 where the size was estimated by PCR analysis. In the case of exon 1, the size given is the number of nucleotides from the A of the ATG to the splice junction, and in the case of exon 10, the size given is the number of nucleotides from the splice junction to the G of the TAG.

(B) Sequence of the intron-exon junction regions. Intron sequences are given in uppercase letters, and exon sequences are given in lowercase letters. The numbers in parentheses correspond to the nucleotide coordinates of the coding sequence present in each exon, assuming that the A of the ATG is nucleotide 1. In the case of exon 1, the sequence upstream of the ATG is sequence derived from genomic clones that correspond to the longest 5'-RACE sequence obtained. The complete DNA sequences used to construct this figure have been submitted to Genbank.
Generation of MSH6-Deficient Mice

(A) Gene targeting strategy. Schematic representation of the gene modification strategy. The organization of the wild-type gene, the targeting construct, and the structure of the locus following gene targeting are shown. Exons are shown as closed boxes. The location of PCR primers for detecting gene targeting events as well as the expected DraI digestion products that will be recognized by the probe are shown.

(B) Southern blot hybridization of DNA from mice from F2 generation. DNA was digested with DraI and hybridized with probe shown in (A). The 4.1 kb band corresponds to wild-type, and the 2.5 kb band corresponds to the modified allele. (1/1), wild-type; (1/2), heterozygous; (2/2), homozygous.

(C) Detection of Msh6 transcript by Northern blot analysis. Total RNA from cell lines of different genotypes was fractionated and blot-hybridized with the probe shown in Figure 2. A β-actin-specific gene probe was used as a control. (1/1), wild-type; (1/2), heterozygous; (2/2), homozygous.

(D) Detection of MSH6 protein by Western blotting. Total protein from cells of different genotypes was fractionated, and Western blot analysis was conducted with an anti-GTBP antibody. The 160 kDa band corresponds to the native MSH6 protein. (1/1), wild-type; (1/2), heterozygous; (2/2), homozygous.

its genomic structure and determined its pattern of expression.

The deduced genomic structure and the sequences at the intron-exon junctions of the gene are shown in Figure 1. Northern blot analysis with the mouse Msh6 probe revealed a single transcript (4.4 kb) in all mouse tissues tested (heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis), with the highest level of expression being observed in testis. Corradi et al. (1996) detected an Msh6-related sequence on mouse chromosome 4, whose functionality is unknown.

Generation of Mice with a Modified Msh6 Gene

The gene targeting vector and the scheme to generate mice with a mutation in the Msh6 gene is shown in Figure 2. The gene targeting vector, designated pMSH6NTK, contains a PGKne expression cassette inserted into the Eco47III site in the fourth exon of the gene. This site corresponds to codon 336, and sequencing of the junction revealed that the insertion results in a stop codon 21 nucleotides downstream. A correct gene targeting event would result in a truncated protein, if any, and would lack the critical conserved functional motifs that are located at the COOH end of the normal protein (Drummond et al., 1995). Therefore, we expected the targeting event to result in a null mutation. pMSH6NTK was linearized and introduced into ES cells. We isolated 192 G418- and ganciclovir-resistant ES cell colonies and screened them for the homologous recombination event by a PCR-based assay. We found that 3 out of the 192 ES cell colonies contained the appropriately modified Msh6 locus. The targeting event was verified by Southern blot analysis.

All three of the appropriately targeted cell lines (Msh6-104, -141 and -147) were injected into blastocysts derived from C57BL/6 females. All of the cell lines yielded mice with high degrees of chimerism, as determined by coat color, and several of the chimeras derived from each of the three cell lines transmitted the modified chromosome through their germline.

To rapidly generate cells that are homozygous for Msh6 gene mutation, we made a gene modification construct that was essentially identical to pMSH6NTK except for the replacement of PKGneo with PGKhygro (pMSH6HTK). Transfection of the pMSH6HTK into Msh6-104, a cell line that had one modified copy of Msh6, yielded several colonies in which both copies of Msh6 were modified by gene targeting.

Homozygous Msh6 Mutant Animals Are Viable

To determine the viability of animals that are homozygous for the Msh6 mutation, heterozygous F1 mice were interbred and the F2 offspring genotyped by PCR and Southern blot analysis. DNA was digested with DraI, fractionated, and blot hybridized with a probe (Probe A, Figure 2) corresponding to the 3' end of exon 4 that is not present in the targeting vector. This probe recognizes a 4.1 kb fragment from the wild-type allele and a 2.5 kb fragment from the modified locus. The probe also recognizes a 4.4 kb band, presumably corresponding to the
have any MSH6 protein. To confirm these expectations, 1 from mutant ES cells suggested that they would not tion in the

Lines

Wild-type (Msh6 transcript was detectable from reduced levels, was detectable in 1, 2, or 4 extra nucleotides.

locus and the lack of any detectable Msh6 message We examined the survival of animals that carry a muta-

Msh6-related sequence on chromosome 4. Representa-

tive results of this analysis are shown in Figure 2B. We ex-

Msh6 Mutant Cells Do Not Produce a Stable Transcript or Protein

We ascertained if the mutant cells produced stable Msh6 transcripts. Total RNA from two double-targeted ES cell lines was analyzed by Northern blotting with probe A shown in Figure 2. Results of this analysis are shown in Figure 2C. The RNA from /+ ES cells contained a 4.4 kb transcript. A similarly sized transcript, although at reduced levels, was detectable in +/− cells, while no transcript was detectable from Msh6 mutant cells.

The nature of the mutation introduced into the Msh6 locus and the lack of any detectable Msh6 message from mutant ES cells suggested that they would not have any MSH6 protein. To confirm these expectations, we isolated total protein from the cells and analyzed it by Western blotting with an MSH6-specific antibody (Palombo et al., 1995). Results from this experiment are shown in Figure 2D. We were able to detect the expected 160 kDa protein in extracts from /+/ and +/− cells but not in −/− cells.

Msh6 Mutant Mice Have a Significantly Reduced Life Span

We examined the survival of animals that carry a mutation in the Msh6 gene. We maintained 33 wild-type, 40 +/−, and 28 −/− animals in identical conditions and recorded the time when they became moribund or the time of natural death. A summary of these observations is presented in Figure 4. None of the three classes of mice died during the first 3 months of life. At 9 months after birth, 97%±100% of the /+ and +/− animals were alive, while only 60% of the −/− animals were alive. By

Figure 3. Mismatch Repair Efficiency in Extracts from Mouse Cell Lines

Wild-type (+/+/), heterozygous (+/−), and homozygous (−/−) Msh6 cell extracts were used to examine the efficiency of repair of substrates containing a G-G mismatch at position 88 or a C-A mismatch at position −11. The nick was located at base pair −264 (3′ nick) or at position +276 (5′ nick), where +1 is the first transcribed base of the lacZ gene in M13mp12. Frameshift heteroduplexes contained 1, 2, or 4 extra bases in the (−) strand, at nucleotides 91, 90–91, or 68–71, respectively. Reactions were incubated for 15 min. The results are based on counting several hundred plaques per variable. In all cases where repair was observed, the change in the ratio of blue to colorless plaques indicates that repair occurred in the strand containing the nick, which is known to serve as a strand-discrimina-

tion signal in vitro.

Figure 4. Survival of Msh6 Mutant Mice

The time of death or the time when mice became moribund was recorded, and the survival curves were generated by using the Prism (GraphPad Prism 2.0) software package. The differences between the −/− versus +/− and +/− curves are significantly different (P < 0.0001 according to the log rank test).

Extracts of Msh6−/− Cells Have Impaired Mismatch Repair Activity

Extracts of human tumor cell lines having mutations in MSH6 are defective in repair of base-base mismatches but retain some ability to repair insertion/deletion mismatches (Umar et al., 1994; Boyer et al., 1995; Drummond et al., 1995). However, unequivocally assigning the mismatch repair defect in these cells to the MSH6 mutation is made difficult by the fact that they are derived from tumors and may contain mutations in other genes. Extracts from mouse ES cells homozygous for Msh6 mutations are ideally suited for ascertaining the role of MSH6 in mismatch repair. Thus, extracts were prepared from the homozygous ES cell line MSH6-NH14 as well as from /+ and +/− cells. These were tested for their ability to repair substrates containing different mismatches. Extracts prepared from Msh6−/− cells did not catalyze repair of a G-G mismatch in which the nick, which serves as the signal for strand specificity, is located either 3′ or 5′ to the mismatch (Figure 3). Similarly, no repair of an A-C mismatch (3′ nick) was detected in an extract of −/− cells. In contrast, repair of these same substrates was observed in extracts of both /+ and +/− cells. Extracts of all three cell types repaired substrates containing either 1, 2, or 4 extra nucleotides in one strand (Figure 3). These data show that MSH6 is required for strand-specific repair in vitro of single base-base mismatches but is not essential for repair of insertion/deletion mismatches involving either 1, 2, or 4 extra nucleotides.
MSH6-Deficient Mice

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Figure 5. Tumors Observed in Msh6 Mutant Mice

(A) A tumor of the small intestine (size bar = 2 mm).
(B) Section of adenocarcinoma stained with H, E, and an APC-specific antibody. Note the brown color that represents APC protein expression and its lack (arrow) in the tumor (size bar = 80 μm).
(C) Mesenteric lymphoma (size bar = 10 mm).
(D) Section of lymphoma stained with a B cell-specific antibody (size bar = 20 μm).
(E) Hepatoma (size bar = 2 mm).
(F) Section of hepatoma stained with H and E (size bar = 40 μm).

12 months of age, 94% of the +/+ and 90% of the +/- animals were alive, while only 35% of the +/- animals were alive. At 15 months of age, only 8% of the +/- mice were alive, while more than 80% of the +/+ and +/- mice were alive. These results suggest that lack of Msh6 gene expression is not consistent with a normal life span. It should be noted that the median survival time of MSH2-deficient mice is 5-6 months (Reitmair et al., 1996), in contrast to the 10 months median survival time of MSH6-deficient mice.

Msh6 Mutant Mice Develop a Spectrum of Tumors

Heterozygous and homozygous Msh6 mutant mice were examined for the development of tumors. We followed the health of a total of 33 ++, 40 +/-, and 28 +/- mice. Over a 15 month period, 28 of 33 (85%) ++ and 31 of 40 (78%) +/- mice were healthy. We sacrificed 5 +/- mice that became moribund and found them to bear easily identifiable tumor masses. Among the homozygotes, 14 mice (50%) died prematurely and were not available for detailed analysis. 13 of the mice were sacrificed because of morbidity. Of these, 12 had a spectrum of relatively large tumors in a number of different locations. One mouse, which is more than 15 months old, is still alive. A representative sample of tumors that were observed is shown in Figure 5, and the complete data are summarized in Table 1. In heterozygous mice that had tumors, the most frequently found tumor was non-Hodgkin’s lymphoma (NHL) followed by tumors in the gastrointestinal (GI) tract. In homozygous mice, the GI tract tumors as well as the lymphomas were frequent. In addition, these mice had tumors of the liver, lung, skin, fibrous, and endothelial cells. An interesting feature that was observed in two different +/+ and one +/- mice was the presence of two aggressive tumor types adjacent to each other. This type of tumor, referred to as a collision tumor, consisted of NHL and early invasive carcinoma of duodenum and jejunum. Gross examination of 14- to 16- month old +/+ mice (n = 10) did not reveal any tumors.

The Lymphomas Are Invasive and Are of B- and T-Cell Origin

We observed that among the 18 (5 ++, 13 +/-) mice that were examined, 2/13 homozygotes and 2/5 heterozygotes had a significantly enlarged spleen. This enlargement is due to the infiltration of the NHL. We also observed that the NHL was invasive in several of the cases and was detected in the heart, liver, lung, thymus, uterus, and several parts of the GI tract. To ascertain the cellular origin of the lymphomas, sections of the tumors were stained with CD45R/B220, a B cell-specific antibody and two CD3 antibodies that are T cell-specific. Lymphomas from 4 ++ and 8 +/- mice were examined by immunotyping. Of these, 9 were determined to be B-cell lymphomas (Figure 5) that could be classified into 3 diffuse large cleave, 3 non-large cleave, 1 immunoblastic, 1 large cleave-immunoblastic, and 1 large cleave-mixed cell type. The other 3 were T-cell lymphomas that were classified as lymphoblastic lymphomas.

Tumors in Msh6 Mutant Mice Do Not Show Microsatellite Instability

The microsatellite instability seen in tumors from HNPCC patients appears to result from a failure to repair insertion/deletion mispairs, and such microsatellite instability
Figure 6. Comparison of Microsatellite Instability in Tumors from Mutant Msh6 and Mlh1 Mice

Tumor DNA was tested for microsatellite instability as described in the Experimental Procedures.

(A) Test with marker JH104, a mononucleotide repeat marker located in intron 1b of the Msh3 gene.

(B) Test with D7Mit91, a dinucleotide repeat marker. (WT), wild type tail DNA. (T1)±(T3) refer to tumors. (*) shows an altered size band.

shows a high degree of correlation with the presence of germline Msh2 and Mlh1 mutations that cause defects in repair of insertion/deletion mismatches (Kolodner et al., 1995; Kolodner, 1996; Liu et al., 1996; Modrich and Lahue, 1996). Because repair of insertion/deletion mismatches was observed in extracts from Msh6\(^{-/-}\) cells, it seemed likely that the Msh6 mutant tumor cells would not show a high incidence of microsatellite instability. To test this prediction, DNA from 12 different tumors derived from 6 different mice was obtained and used for PCR reactions with 8 different sets of PCR primers. For comparison, we used tumors derived from mice homozygous for Mlh1, another mismatch repair gene (Edelmann et al., 1996). Four of these (D1MIT36, D7MIT91, D14MIT15, and D18MIT15) primers amplify sequences containing dinucleotide repeats, and the other four (JH101, 102, 103 and 104) amplify sequences containing mononucleotide repeats. The sizes of the alleles amplified by each of these markers in the 129/Ola and C57Bl/6 strain are known. Representative results are shown in Figure 6. Testing of 4 separate tumors from Mlh1 mutant mice with 5 different markers revealed microsatellite instability of 45% with instability being observed at both mono- and dinucleotide repeat loci. A similar test with tumors from Msh6 mutant mice revealed 2/96 (2.1%) instability, and the unstable loci observed were dinucleotide repeat loci. Our results show that the tumors present in Msh6 animals show little or no microsatellite instability.

GI Tumors in Mutant Mice Do Not Have the Adenomatous Polyposis Coli (APC) Protein

Mutations in the APC gene are responsible for FAP, an inherited cancer predisposition syndrome characterized by progressive development of colonic polyps and tumors and for a large number of sporadic colorectal tumors. The tumors in HNPCC individuals also have mutations in APC (Lazar et al., 1994; Huang et al., 1996), suggesting that the hypermutability seen in these tumors affects the APC gene. To assess if the GI tumors seen in Msh6 mutant mice have a similar basis, we examined the tumors for the expression of the APC protein using anti-APC antibodies. We examined 6 such tumors in Msh6\(^{-/-}\) mice. The APC protein was detected in the cytoplasm of normal epithelial cells of the villi in the
Discussion

Inactivation of the Mouse Msh6 Gene

To examine the role of the mismatch repair gene, Msh6, in normal growth and development, we generated mice carrying a mutation in the Msh6 gene. Several lines of evidence show that we have inactivated the only functional Msh6 gene in the mouse genome. Southern blot analysis of DNA from gene-targeted cells and mice derived from them revealed that they contained the desired gene modification. Northern blot analysis revealed no Msh6 transcript in Msh6−/− cells and a reduced level of message in Msh6+/− cells. Western blot analysis with hMSH6 antibodies detected the expected 160 kDa protein from Msh6+/+ and +/− cell extracts, but no such protein was detectable in Msh6−/− cells. These results provide convincing evidence that the Msh6 gene was functionally inactivated by the gene targeting event and that we have mice that are MSH6-deficient.

Extracts of Msh6−/− Cells Cannot Repair Base-Base Mismatches in DNA

Extracts of Msh6−/− ES cells were completely defective in their ability to repair base-base mismatches that are repaired in +/+ and +/− extracts (Figure 3). This is consistent with a model in which MSH6 partners with MSH2 to form a heterodimer that recognizes and binds to single base-base mismatches to initiate repair (Drummond et al., 1995). The ability of the −/− extract to repair 1, 2, and 4 nucleotide insertion/deletion mismatches (Figure 3) is consistent with the existence of a second mispair recognition complex in these cells that is either solely responsible for recognition of insertion/deletion mispairs or partially redundant with the MHS2-MSH6 complex for recognition of insertion/deletion mispairs. Based on the analysis of the Msh2, Msh3, and Msh6 genes and gene products in yeast and human systems, this other complex is likely to consist of the MSH2 and MSH3 proteins (Drummond et al., 1995; Achara et al., 1996; Habraken et al., 1996; J ohnson et al., 1996; Marsischky et al., 1996; Palombo et al., 1996; Sia et al., 1997). The in vitro mismatch repair defects we have observed in our analysis of Msh6 mutant extracts are essentially as predicted from the analysis of the mutator phenotypes caused by Msh6 mutations in yeast (Marsischky et al., 1996; Sia et al., 1997). It is also consistent with the observation of a lack of dinucleotide repeat instability and reduced mononucleotide repeat instability in Msh6 mutant tumor cell lines and the lack of other mononucleotide repeat or dinucleotide repeat instability in the tumors from Msh6 mutant mice (Bhattacharyya et al., 1994; Shibata et al., 1994; Papadopoulos et al., 1995). The mismatch repair activity data in Figure 3 are also consistent with results obtained with a tumor cell line that is mutant in both Msh6 and Msh3. Extracts of this cell line fail to repair base-base mismatches or 1-, 2-, and 4-nucleotide insertion/deletion mismatches (Risinger et al., 1996). However, upon transfer of either chromosome 2 encoding MSH2 and MSH6 or chromosome 5 encoding MSH3, extracts from both MSH6 (−) and MSH3 (+) MSH6 (−) cells repaired 1-, 2-, and 4-nucleotide insertion/deletion mismatches, while only the extract from MSH3 (−)MSH6 (+) cells repaired base-base mismatches (Umair et al., submitted). These data support the view that the primary defect in Msh6 mutant cells is the loss of the ability to repair base-base mispairs and that the mutations that accumulate in Msh6 mutant mammalian cells should primarily be base substitution mutations.

Cell lines that are exclusively defective in Msh6 have not been described. The cell lines that we have generated have such a defect and may prove to be valuable in assessing the status of the mismatch repair complexes in the absence of the MSH6 protein.

Msh6 Mutation Leads to Tumor Susceptibility

Mutations in MSH6 have been observed in a small number of human colonic tumor cell lines. However these mutations have often been observed in conjunction with mutations in other mismatch repair genes that could conceivably contribute to or be the cause of the mutator phenotype seen in these cell lines (da Costa et al., 1995; Malkhosyan et al., 1996; Risinger et al., 1996). Although Drummond et al. (1995) suggested that mutations in MSH6 may result in hypermutability and a predisposition to cancer, no germline mutations in MSH6 have been observed in HNPCC families (Liu et al., 1996; Peltomaki et al., 1997). These data suggested that MSH6 may play a role in any role in tumor suppression. The studies presented here show that mice lacking MSH6 have a predisposition to develop lymphomas and a number of other tumors.

Based upon the mutator phenotype seen in Msh6 mutants and the proposed mispair recognition properties of the MSH2-MSH6 complex, absence of the gene product is expected to lead to accumulation of point mutations (Marsischky et al., 1996). When the appropriate target genes are mutated, the resulting mutant cells would be expected to enter a proliferative pathway. Based upon this notion, lack of MSH6 would be expected to lead to reduced life span and tumorigenicity of a number of different cell types. Both of these expectations are realized in Msh6 mutant mice. The precise targets for mutations for the development of lymphomas in Msh6 mice are not known. It is of interest, however, to note that lymphomas are also a predominant early tumor in Msh2 mutant mice (Reitmair et al., 1996). Our analysis of the colonic tumors that develop in the Msh6 mutant mice shows that they have lost APC expression. APC gene mutations have been implicated in FAP and in sporadic colorectal tumors and colorectal tumors in HNPCC patients, leading to the proposal that the acquisition of APC gene mutations plays an important early step in the development of these tumors. It is attractive to propose that mutation of the Apc gene due to the...
mutator phenotype caused by Msh6 mutations also plays an important role in the development of the GI tumors we observed.

Mutations in another mismatch repair gene, Msh2, also leads to early lymphomas and colonic tumors later in the lives of mice. MSH2 mutations are common in HNPCC patients (Liu et al., 1996; Pełtomaki et al., 1997). Therefore, it is reasonable to ask why germline mutations in MSH6 were not detected in HNPCC families. There are two possible explanations for this. Much of the attention on HNPCC families has been focused on those whose tumors show a replication error (RER+) phenotype as detected by microsatellite instability because many HNPCC families show this phenotype (Altonen et al., 1993; Lindblom et al., 1993). Such microsatellite instability appears to result from failure to repair insertion/deletion mispairs, a type of repair that does not require MSH6 (Marsischky et al., 1996; Johnson et al., 1993; Lindblom et al., 1993). Such microsatellite instability would be expected to exclude from analysis those families that have germline MSH6 mutations. Alternately, the mutator phenotype caused by MSH6 mutations is quite different from the mutator phenotype caused by MSH2 or MLH1 mutations, and it is possible that the MSH6 mutator phenotype does not result in the inactivation of the target genes required for cancer predisposition. Our results demonstrate that MSH6 germline mutations result in cancer susceptibility, supporting the view that HNPCC or other families having MSH6 germline mutations could have been excluded from analysis by selection of those with an RER+ phenotype. We predict that HNPCC families that do not show an RER+ phenotype or other types of families that have a predisposition to lymphomas as well as GI tumors are sterile, while the females are normal. Both males and females deficient in MLH1 are sterile. In both cases, the sterility is the result of meiotic abnormalities. Based on our results with Mlh1-deficient mice (Edelmann et al., 1996), we suggested that in meiosis, Msh6 might be replaced by another member of the Msh gene family. The fact that Msh6-deficient mice are fertile suggests that Msh6 is dispensable in meiosis. It will now be of importance to ascertain if other members of the Msh gene family function in meiosis by determining if mutations in these genes cause the same types of meiotic phenotypes as Pms2 or Mlh1 mutations.

Experimental Procedures

Isolation and Characterization of the Mouse Msh6 Gene

The isolation and characterization of mouse Msh6 cDNA clones, 5’-RACE products, and P1 clones containing the Msh6 genomic region has been performed essentially as described in previous studies (Fishel et al., 1993; Bronner et al., 1994; Kolodner and Alani 1994; Kolodner et al., 1995). Degenerate PCR was first used to isolate the first 5’ end of the mouse Msh6 coding sequence. Specifically, primed cDNA was first made from S13 RNA using the degenerate primer 5’-GTTCCCTG/CAT/GCC/ATG/TGG/GIG/AA and the resulting PCR product was cloned into the EcoRV site of pBluescript SK(-) and sequenced. A hybridization probe made using the resulting partial Msh6 clone was used to screen a mouse pre-B cell, size-selected cDNA library in the lambda gt10 vector, and 26 positives were obtained. Clones containing the longest inserts were identified by PCR with primers 20322 5’T-TTAGCAATTGCAGCTCGG and 20068 5’-CATTAGATTTATCTTCCAGG, and the PCR product corresponding to the longest insert was sequenced. Additional 5’ sequence was then obtained using 5’ RACE using mouse spleen double-stranded cDNA containing a 5’ adaptor (Clonetech), primer 5’-CATCCAATATACGACTCCTACTTATAGGCG matching the 5’ adaptor, and a Msh6-specific primer 5’-GCGGGCTGCTCCGCTAGGGCCCGCCGCGCCGCCCGCGCCCGC derived from the 5’ end of the cDNA sequence. The resulting RACE product was cloned into the EcoRV site of pBluescript SK(+), and sequenced. A hybridization probe made using the resulting partial Msh6 clone was used to screen a mouse P1 library (Genome Systems) using primers 23038 5’-GAGTCCTGCTTTCCAGG and 23041 5’-GTACGAAAGAACCGACATAGGC and 2 clones (numbers 8229 and 8230) were obtained. The intron-exon junctions, all of the exons, and many of the introns were sequenced directly from P1 DNA isolated from a C57BL/6 strain and primers derived from the coding and intron sequences using procedures provided by Genome Systems essentially as previously described. All DNA sequencing was performed with an ABI/Perkin Elmer 373 sequencer and dye terminator chemistry and contigs were constructed with Sequencer software.

Construction of the pMSH6 Targeting Vectors

A genomic Msh6 fragment containing the exons 2, 3, and a portion of exon 4 was obtained by screening a mouse genomic Charon 35, 129/Ola phage library with a full-length mouse Msh6 cDNA probe. A 7.5 kb SalI fragment was subcloned into pUC19, and the intron-exon structure of part of the gene was determined using sequencing and restriction mapping (Figure 1). A 1.7 kb Xhol-HindIII PGKneo was blunted with Klenow polymerase and cloned into the EcoRI site at codon 336 in exon 4. Finally, a 1.1 kb Xhol-BamHI fragment
from pMC1tk (Mansour et al., 1988) was treated with Klenow polymerase and cloned into the single Smal restriction site located 3’ to exon 4. The resulting gene targeting clone was designated pMSH6NTK. The targeting vector pMSH6HHTK was used for the generation of double knockout ES cell lines identical to pMSH6NTK except that a 2.0 kb Clal-XhoI PGKhygro fragment was cloned into the Eco47III site.

Electroporation of Embryonic Stem Cells
The targeting vector pMSH6NTK (40 μg) was linearized at the single Sse8387 site and electroporated into 2.0 × 10^6 Embryonic stem cells (Joffe et al., 1995) and selected with G418 (150 μg/ml) and ganciclovir (2 μM) as described previously (Sirotnik et al., 1995). Colonies were picked after 10 days and their DNA was screened by PCR using forward primer A 5’-TGGAGAGATTGGAGCTACC-3’ and reverse primer B 5’-AGGCGGTATGGCGAAATAC-3’. The reaction was performed in a 50 μl reaction mixture containing 100 ng of DNA, 5 ng/ml of each primer, 1.5 mM MgCl2, 0.2 mM of each dNTP, and 0.5 U of Taq polymerase. Cycling conditions were: 5 min at 94°C (1 cycle), 1 min at 94°C, 1 min at 57°C, 1.5 min at 72°C (40 cycles), 5 min at 72°C (1 cycle). Positive ES cell colonies were identified by a 1.6 kb PCR fragment specific for the targeting event. Three positive cell lines, MSH6-104, MSH6-141, and MSH6-147, were identified, and the correct targeting event was shown by DraI GTBP antibody. We also thank Marie Lia and Harry Hou, Jr. for these cell lines.

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Generation of MSH6-Deficient Mice
Chimeric mice were generated by injecting C57Bl/6 blastocysts with Aaltonen, L.A., Peltomaki, P., Leach, F., Sistonen, L.P., Albertson, G.D., and Carcinoma arising from DNA mismatch repair deficiency is expressed in percent as 100 × (1 – the ratio of the percentages of mixed bursts obtained from extract-treated and untreated samples).

Analysis of Tumors
Animals were sacrificed by neck dislocation. Tumors, if found, and the gastrointestinal tract and other organs including the lungs, heart, liver, kidneys, and spleen were removed and fixed in 10% neutral buffered formalin. The gastrointestinal tract was opened and examined under a dissecting microscope for tumors. Representative tissues from the tumors and organs were taken for processing and paraffin embedding. All tissue sections were prepared for Hema toxlyn and Eosin stain. Tumor tissues were studied for APC protein expression and lymphoma immunotyping. The antibodies used in this study include the APC proteins C-20 and N-15 (Santa Cruz), B lymphocyte CD45R/B220 (Phar mingen), and T lymphocyte CD3 (Vector and Zymed).

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References
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GenBank Accession Numbers

The genomic sequences defining the Msh6 gene structure and coding sequences have been deposited in GenBank under accession numbers AF031085, AF031086, and AF031087.

Note Added in Proof

As predicted from the work described here, a germline mutation in the human MSH6 gene was detected in affected members of a family with atypical HNPCC (Akiyama et al., 1997, in Cancer Res. 57, 3920-3923). We have also detected a germline nonsense mutation in exon 4 of a HNPCC patient from a family meeting the Amsterdam Criteria whose tumors showed low mononucleotide repeat instability and no dinucleotide repeat instability. We are currently analyzing additional candidate HNPCC families for the presence of germline hMSH6 mutations.