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Mutation in the Mismatch Repair Gene *Msh6* Causes Cancer Susceptibility

Winfried Edelmann,[1,10] Kan Yang,[5] Asad Umar,[6] Joerg Heyer,[2] Kirkland Lau,[1] Kunhua Fan,[5] Wolfgang Liedtke,[3] Paula E. Cohen,[4] Michael F. Kane,[7,8] James R. Lipford,[7] Nianjun Yu,[9] Gray F. Crouse,[9] Jeffrey W. Pollard,[4] Thomas Kunkel,[6] Martin Lipkin,[5] Richard Kolodner,[7,8] and Raju Kucherlapati[2]

[1]Department of Cell Biology
[2]Department of Molecular Genetics
[3]Department of Pathology
[4]Department of Developmental and Molecular Biology
Albert Einstein College of Medicine
Bronx, New York 10461
[5]Strang Cancer Research Laboratory
The Rockefeller University
New York, New York 10021
[6]National Institute of Environmental
Health Sciences
National Institutes of Health
Research Triangle Park, North Carolina 27709
[7]Dana Farber Cancer Center
Boston, Massachusetts 02115
[8]Ludwig Institute for Cancer Research
La Jolla, California 92093
[9]Emory University
Atlanta, Georgia 30322

Summary

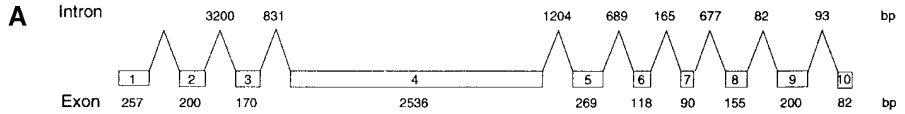
Mice carrying a null mutation in the mismatch repair gene *Msh6* were generated by gene targeting. Cells that were homozygous for the mutation did not produce any detectable MSH6 protein, and extracts prepared from these cells were defective for repair of single nucleotide mismatches. Repair of 1, 2, 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; Peltomaki et al., 1997).

Biochemical studies revealed that a complex of proteins recognize and bind 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

[10]To whom correspondence should be addressed.



B

EXON 1 gcgcggtggatgcgatccttgtaggcgctcggagccgtggcccggtctgcccgtcagccttcatcATGtcccgacaaag (1 to 257) ggccgtgcctcccagGTAGCCGGCTCGGTCGGGGCT
EXON 2 GTTTTTGTTTTTTGATTTTTCTTTTGCAACAGttcttgtgacttctcaccag (258 to 457) gatgttaaagccatatacagGTAAGAGGTAAATGGGGATGG
EXON 3 GTTCTTATTTATATTTCTAGgttcaaagtcaaaggaagc (458 to 627) aggaggaggaggagacagagGTCAGTCACTCCATTCTCTTAG
EXON 4 TACAAGCCCCTTTCTTTGTTTGGCAGgtgcatgaggcttatttatc (628 to 3163) agagtgcattgcagtgttggGTAAGGCTTTGGTTAACCCT
EXON 5 TACTTGTATCCTCTTGACAGatgtcttactgtgcctggct (3164 to 3432) agtctacactcataagacagGTAATTTGTTCTTCATGTTC
EXON 6 CCTTTCTTCCCATCTTCCCAGgctggtctgttggctgtgatg (3433 to 3550) ctcagatagaataatgtcagGTGAGTTGCTTTTCCTCCAC
EXON 7 ACTCAGTTGTTTCTTTTTTTAGgagaaagcacatttttgttg (3551 to 3640) ggtacttgtggatgaattagGTAAGACAGATTTTGTTTGAA
EXON 8 CAGTAATTCCACTTCCACAGgaagaggcaccgcaactttg (3641 to 3795) gtgtgcgcctaggacatatgGTATGTGGAATCAACCAGACA
EXON 9 ATGAACTTTTCTTTCTTGAAGgcatgcatggtagaaaatga (3796 to 3995) cagtcattacagctatttcgGTAATTATATCATAATCTTC
EXON 10 AATGAAATATTTTTTTCCTTTTTTTAAGgaagtttgtctggctactg (3996 to 4077) gctatccataggctgctggccttgattaacggattgTAGactac

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 sizes 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 corresponds to the longest 5′-RACE sequence obtained. The complete DNA sequences used to construct this figure have been submitted to Genbank.

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 resistence 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 ascertained

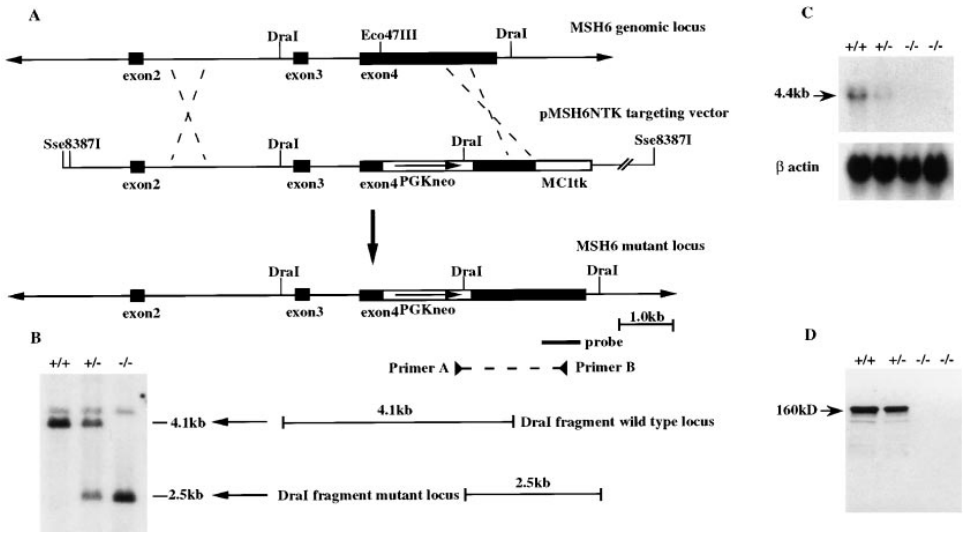


Figure 2. Generation of MSH6-Deficient Mice

(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. (+/+), wild-type; (+/−), heterozygous; (−/−), 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. (+/+), wild-type; (+/−), heterozygous; (−/−) 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. (+/+), wild-type; (+/−), heterozygous; (−/−), 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, contained a PGKneo 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

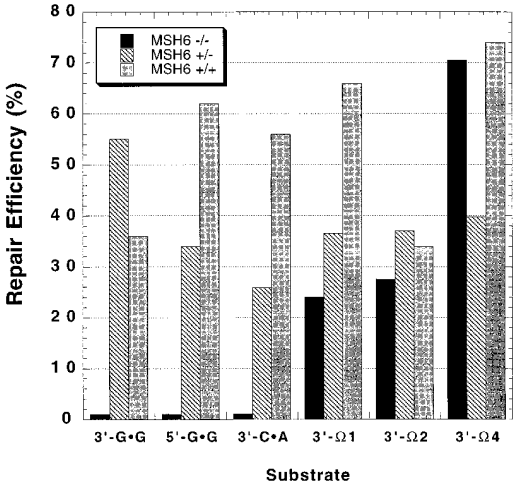


Figure 3. Mismatch Repair Efficiency in Extracts from Mouse Cell Lines

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 M13mp2. 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-discrimination signal in vitro.

Msh6-related sequence on chromosome 4. Representative results of this analysis are shown in Figure 2B. We examined a total of 333 mice from 49 litters. Of these, 107 were wild-type, 142 were heterozygous, and 84 were homozygous for the mutant allele. These results show that a mutation in the *Msh6* gene is consistent with viability of the mice.

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.

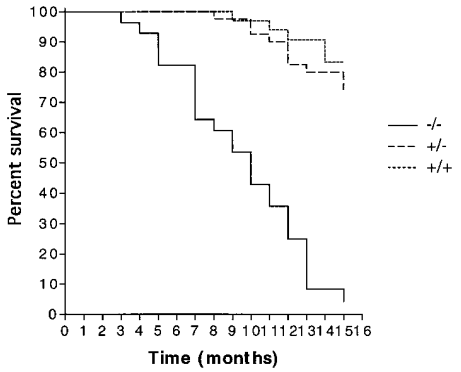


Figure 4. Survival of *Msh6* Mutant Mice

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 +/+ cells 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$^{-/-}$ 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

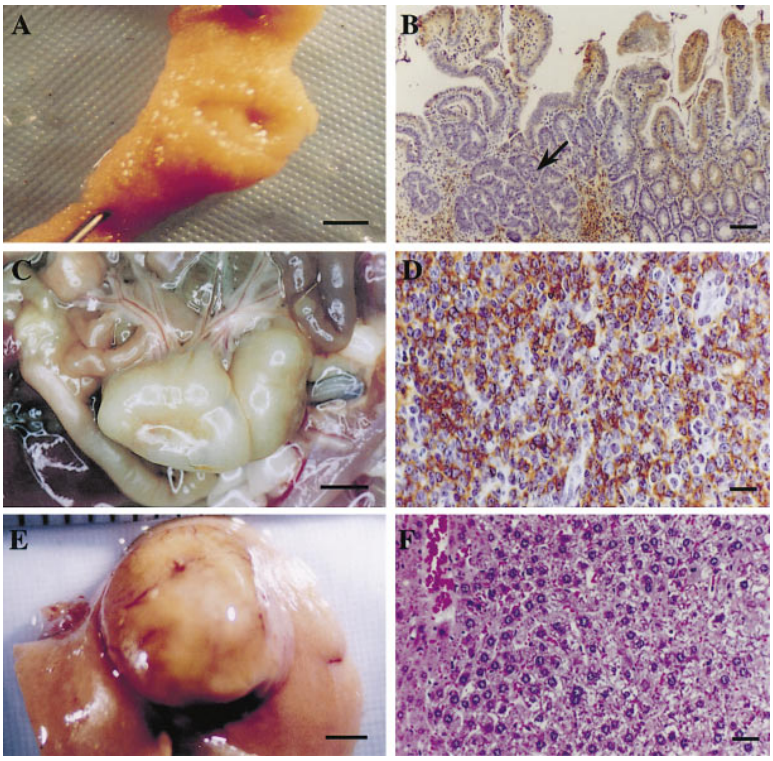


Figure 5. Tumors Observed in *Msh6* Mutant Mice

(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 (Table 1).

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

Table 1. Histopathological Findings in *Msh6* Mutant Mice

Mouse ID (Genotype)	Sex	Age (Months)	Tumors in GI tract	Histologic Type	Lymphomas (NHL, D) Immunologic Type	Involvement	Other Tumors
01 (+/−)	M	11	—	Large cleave–immunoblastic	B cell	Mes, Liv, Thy	—
02 (+/−)	M	11	—	Large noncleave	B cell	Mes, Liv, Thy, Sp, PC	—
03 (+/−)	M	14	CA (Je), ECA/NHL (Duo, IL)	Large noncleave	B cell	Mes, Sp	—
04 (+/−)	F	8	MA (R)	—			Dermatofibroma
08 (+/−)	M	12	—	Immunoblastic	B cell	Mes, Head, Neck	Hemangioma/NHL
05 (−/−)	M	12	CA (Je), ECA (Je), FAD	—			Hepatoma
09 (−/−)	F	10	ND	Large cleave	B cell	Mes	—
10 (−/−)	M	12	CA (Je), A (Je)	—			Hepatoma, Lung CA
11 (−/−)	M	12	—	—			—
12 (−/−)	M	13	ECA (Duo)	—			—
13 (−/−)	M	7	—	Lymphoblastic	T cell	Liv, Duo	—
15 (−/−)	M	14	—	—			Hepatoma
16 (−/−)	M	13	—	Large noncleave	B cell	Mes, Thy	Skin tumors
17 (−/−)	M	13	A (Duo, Je)	Large cleave	B cell	Mes, Sp	—
18 (−/−)	F	6	—	Lymphoblastic	T cell	Mes, Mm, Heart, Lung, Uterus	—
19 (−/−)	M	10	ECA/NHL (Je)	Lymphoblastic	T cell	Mes, Liv, Sp, Sk	—
20 (−/−)	M	15	—	Large cleave	B cell	Mes	—
21 (−/−)	M	11	—	Large cleave–mixed cell	B cell	Mes, LN	—

Abbreviations: Mes, Mesentery; Sp, Spleen; Liv, liver; Thy, Thymus; LN, peripheral lymph node, Duo, duodenum, Je, jejunum; Il, ileum; PC, proximal colon; R, rectum; Mm, mammary gland; Sk, skin; NHL, non-Hodgkin's lymphoma, D, diffuse; MA, microadenoma; CA, invasive adenocarcinoma; ECA, early invasive adenocarcinoma; A, adenoma; FAD, focal area of dysplasia; ND, not done.

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 presense of germline *Msh2* and *Mlh1* mutations that cause defects in repair of insertion/deletion mispairs (Kolodner et al., 1995; Kolodner, 1996; Liu et al., 1996; Modrich and Lahue, 1996). Because repair of insertion/deletion mispairs 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

small intestine and at reduced levels in the crypt cells, while the tumor tissue had little or no detectable APC protein (Figure 5). These results combined with the knowledge about the action of *Msh6* suggest that mutations in the *Apc* gene with a concomitant absence or reduction of the APC protein may be causal in the development of the GI tumors. Molecular biological tests are necessary to unambiguously address this issue.

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; Acharya et al., 1996; Habraken et al., 1996; Johnson 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 is exactly 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 either mononucleotide repeat or dinucleotide repair 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 MSH3 (−) 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 (Umar 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 little if 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 aquisition 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; Peltomaki 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 (Aaltonen 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., 1996; Sia et al., 1997). Thus, the selection of such RER^+ HNPCC families 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 excellent candidates for having germline mutations in the *MSH6* gene. Efforts to identify such families are underway.

Roles of MSH6 and MSH3

Based on the phenotypes observed in yeast and based on in vitro interactions involving MSH3 and MSH6, it has been suggested that these two proteins would be capable of substituting for each other and that a double mutant of *Msh3* and *Msh6* is required to elicit a phenotype that would be equivalent to the knockout of *Msh2* (Johnson et al., 1996; Marsischky et al., 1996; Sia et al., 1997). Experimental results described here show that the absence of MSH6 cannot be fully compensated by MSH3 because *Msh6* mutations clearly predispose mice to the development of cancer. The fact that *Msh6* mutant mice have a longer life span than *Msh2* mutant mice and that they develop tumors somewhat later in life may suggest a small compensatory role for *Msh3*. A better understanding of the relative roles of *Msh3* and *Msh6* would require development of mice that lack *Msh3*. We have generated such mice and are examining the phenotypes caused by *Msh3* mutations by themselves and in combination with *Msh6* mutations. Such mice might also help us to better understand the relative roles of MSH3 and MSH6 in mammalian mismatch repair.

Different Mismatch Repair Genes Play Different Roles in Mice

Two sets of mice with mutations in a member of the *Msh* gene family (*Msh2* and *Msh6*) and two sets of mice with mutations in a member of the *Mlh* gene family (*Mlh1* and *Pms2*) are now available. MSH2- and MSH6-deficient mice are viable and fertile and show a cancer predisposition phenotype (Reitmair et al., 1995, 1996; present work). The *Mlh1* and *Pms2* mice are also viable in the homozygous state and show a predisposition to cancer but have varying degrees of sterility (Baker et al., 1995, 1996; Edelmann et al., 1996). The PMS2 males 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, *Msh2* 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 a portion of the mouse *Msh6* coding sequence. Specifically, primed cDNA was first made from 3T3 RNA using the degenerate primer 5′-IGTICCIC(T/G)ICCIAI(T/C)TC(G/A)TC and Superscript RT (Life Technologies). This cDNA and primer were then used in PCR reactions with Taq DNA polymerase and a second degenerate PCR primer, 5′-IGGICCIAA(C/T)ATGGGIGGIAA, 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′-TTGAGCAAGTTCAGCCTGG and 20068 5′-CTTATGAGTATTTCTTCCAGG, 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′-CCATCCTAAT ACGACTCACTATAGGGC matching the 5′ adaptor, and a *Msh6*-specific primer 5′-GCCGGCTGCTTCGCCTAGGCCCCTG derived from the 5′ end of the cDNA sequence. The resulting RACE product was cloned into the EcoRV site of pBluescript SK(+) and sequenced. P1 genomic clones containing the *Msh6* gene were obtained by screening a mouse 129 P1 library (Genome Systems) using primers 23038 5′-GAGTCCGGTCTTCCGAGCC and 23041 5′-GTACGAAG AAACGACATACAGC 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 Cre^- host strain with primers derived from the coding and intron sequences using proceedures 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 Sequencher 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 XhoI–HindIII PGKneo was blunted with Klenow polymerase and cloned into the Eco47III site at codon 336 in exon 4. Finally, a 1.1 kb XhoI–BamHI fragment

from pMC1tk (Mansour et al., 1988) was treated with Klenow polymerase and cloned into the single SmaI restriction site located 3′ to exon 4. The resulting gene targeting clone was designated pMSH6NTK. The targeting vector pMSH6HTK used for the generation of double knockout ES cell lines was identical to pMSH6NTK except that a 2.0 kb ClaI–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 Sse8387I site and electroporated into 2.0×10^7 WW6 embryonic stem cells (Ioffe et al., 1995) and selected with G418 (150 μg/ml) and ganciclovir (2 μM) as described previously (Sirotkin et al., 1995). Colonies were picked after 10 days and their DNA was screened by PCR using forward primer A 5′-TGGAAGGATTGGAGCTACGG-3′ and reverse primer B 5′-AGGGCGTATGGCAAGTATCC-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 $MgCl_2$, 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 digestion of high molecular weight DNA and Southern Blot analysis using a 680 bp probe directed at the 3′ region of exon 4. For the isolation of double knockout embryonic stem cell lines, MSH6-104 cells were transfected with MSH6HTK as described and selected with G418 (150 μg/ml), hygromycin (110 μg/ml), and ganciclovir (2 μM). Resistant colonies were picked after 10 days and screened by PCR. The conditions were identical to those described above except that a hygromycin-specific primer A was used: 5′-GAAGTACTCGCC GATAGTGG-3′. Positive colonies were identified by a 1.7 kb PCR fragment specific for the targeting event. Two positive cell lines MSH6-4NH and MSH6-13NH were identified.

Generation of MSH6-Deficient Mice

Chimeric mice were generated by injecting C57Bl/6 blastocysts with 8–12 embryonic stem cells derived from the MSH6-104, MSH6-141, and MSH6-147 colonies. All three cell lines gave rise to male chimeric animals that were mated with C57Bl/6 females. Chimeras obtained from all three cell lines transmitted the *Msh6* mutation through the germline. F1 heterozygotes were interbred to obtain homozygous *Msh6* mutant animals.

Western Blot Analysis

For Western blot analysis, equal amounts of protein from cultured cell extracts were separated on a 10% SDS–polyacrylamide gel and transferred onto an Immobilon-P (Millipore) membrane. The membrane was blocked in TBS, 0.1% Tween-20, 5% nonfat dry milk, 10% donkey serum (Sigma) and incubated with 1:500 diluted primary anti-GTBP antibody (Palombo et al., 1995). Bound protein was detected by chemiluminescence using a donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham).

Microsatellite Instability Analysis

DNA was extracted from tumor tissue and tails and subjected to PCR. Four end-labeled primer pairs were used to amplify sequences containing dinucleotide repeats (D1Mit36, D7Mit91, D14Mit15, and D18Mit15 [Dietrich et al., 1994]) and four others to amplify sequences containing mononucleotide repeats (JH101, JH102, JH103, and JH104; primer sequences were as follows: JH101F, 5′-GTGGTAGAA TACTTGGCTAAC-3′; JH101R, 5′-GCAGACTTGAGACTGTACTTG-3′; JH102F, 5′-CATTTCTCTGGGATCGCCTT-3′; JH102R, 5′-CCCGCCT TTGATTCCTTTGT-3′; JH103F, 5′-TTCATCAGTCTTCTGGCTCC-3′; JH103R, 5′-AGTGGTGATAGCAGCTTAGC-3′; JH104F, 5′-AGGTGA TTGTAACGGGCATC-3′; and JH104R, 5′-TATCCTCTCAGTGGTGA GTG-3′. Amplified PCR products were separated on a denaturing polyacrylamide gel and autoradiographed for analysis.

Cell-Free Extracts and Mismatch Repair Assay

Cell-free extracts were prepared from logarithmically growing cultures as described (Thomas et al., 1995). Procedures for measuring mismatch repair have been described (Thomas et al., 1991, 1995). The reaction products were processed and introduced into E. coli NR9162 (mutS), and plaque colors were determined. Repair efficiency is expressed in percent as $100 \times (1 -$ 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 Hematoxylin 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 (Pharmingen), and T lymphocyte CD3 (Vector and Zymed).

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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 h*MSH6* mutations.