

Multiple Intestinal Neoplasia Caused by a Mutation in the Murine Homolog of the APC Gene

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Germ-line mutations of the APC gene are responsible for familial adenomatous polyposis (FAP), an autosomal dominantly inherited disease in humans. Patients with FAP develop multiple benign colorectal tumors. Recently, a mouse lineage that exhibits an autosomal dominantly inherited predisposition to multiple intestinal neoplasia (Min) was described. Linkage analysis showed that the murine homolog of the APC gene (*mApc*) was tightly linked to the *Min* locus. Sequence comparison of *mApc* between normal and Min-affected mice identified a nonsense mutation, which cosegregated with the Min phenotype. This mutation is analogous to those found in FAP kindreds and in sporadic colorectal cancers.

One or more tumor suppressor genes located on chromosome 5q are important in the development of colorectal cancer (1). Two candidate tumor suppressor genes (APC and MCC), on chromosome 5q21 (2-4), are somatically mutated in sporadic colorectal cancers (2, 5). The APC gene is also mutated in the germ line of patients with FAP, an autosomal dominantly inherited disease that predisposes to colorectal cancer (5, 6). Patients affected with FAP develop hundreds to thousands of benign colorectal tumors during their second and third decades of life. Some of these tumors will progress to cancer if they are not removed. Extracolonic manifestations, such as gastric and small intestinal polyps, osteomas, and desmoid tumors, have been observed in some FAP patients (7).

A previously described mutant mouse lineage that is predisposed to Min may provide a model for colorectal tumorigenesis. This lineage was established from an ethylnitrosourea-treated C57BL/6J (B6) male mouse (8). The Min phenotype is a fully penetrant autosomal dominant trait. Young adult Min mice develop numerous adenomas throughout their intestinal tract. Although the distribution of tumors along the intestine is different from that in human FAP, the fact that both phenotypes are autosomal dominantly inherited and involve multiple intestinal tract tumors raises the possibility that Min results from a mutation in the murine homolog of the APC gene (*mApc*).

To test this possibility, we determined whether *mApc* was linked to the *Min* locus. We screened an adult BALB/c mouse brain cDNA library at reduced stringency with

fragments of the human APC cDNA (9). One murine cDNA clone, *mApc*-51a, corresponding to nucleotides 135 to 1412 of the human APC sequence (10), was then used to investigate linkage between *mApc* and *Min* in segregating backcrosses. Min-affected B6 male mice were crossed to AKR/J (AKR) or CAST/Ei (CAST) females. The Min-affected F₁ mice were then backcrossed to normal B6 mice in each parental orientation. The resulting backcross mice were scored (8) for the Min phenotype (the presence of intestinal tumors). If *Min* is a mutation in *mApc*, then mice in the backcross expressing the Min phenotype would be homozygous for the B6 alleles of *mApc*. Conversely, backcross mice that do not express the Min phenotype would be heterozygous (B6/AKR or B6/CAST).

A Pst I restriction fragment length polymorphism (RFLP) between the B6 and AKR or CAST strains was identified by Southern (DNA) blot hybridization to the *mApc*-51a probe (Fig. 1). This polymorphism was used to analyze 74 mice from the CAST backcross, and no discordance between the inheritance of the B6 *mApc* alleles and the Min phenotype was observed (Table 1). Analysis of 95 mice from the AKR backcross revealed a single discordant animal that was heterozy-

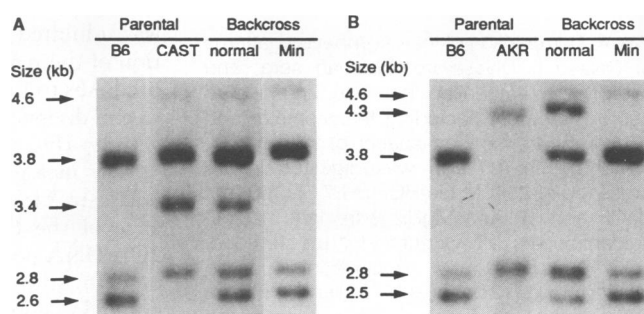
gous but had tumors (Table 1). These results suggest that Min resulted from a mutation in the *mApc* or a closely linked gene.

More complete examination of this discordant mouse revealed that it did not reflect fully the Min phenotype; it had lived much longer than most Min mice and had carried only two duodenal tumors, whereas Min-affected AKR backcross animals had an average of 16 tumors (SD, 12; range, 2 to 50). Sporadic intestinal tumors in mice, especially of the duodenum, have been documented (11) and are the probable source of tumors in this mouse.

We sequenced the entire coding region of *mApc* to identify the mutation in Min mice. To maximize the information obtained in this study, we sequenced the *mApc* cDNA from five mice: a normal B6 mouse, a Min B6 mouse, a normal (B6 × CAST) F₁ mouse, a Min (B6 × CAST) F₁ mouse, and a normal CAST mouse. Two different Min mice were used to identify and confirm any mutation responsible for the Min phenotype. However, it was possible that the *Min* mutation would only affect the amount of *mApc* expression. The three normal mice were included to facilitate identification of nucleotide sequence polymorphisms between B6 and CAST. These polymorphisms could then be used to determine whether both wild-type and mutant alleles of *mApc* were expressed. Total RNA was isolated from brains of the five mice, and randomly primed first strand cDNA was prepared with the use of reverse transcriptase (RT). The polymerase chain reaction (PCR) was then used to amplify (3) the coding region of the cDNA for *mApc*. Fifteen pairs of primers were used to amplify the coding region of *mApc* from the cDNA (12). Each PCR product was then cloned, and pools of clones were sequenced with internal primers (13). The entire coding region of *mApc* and 27 base pairs (bp) of the 3' untranslated region was sequenced.

The *mApc* transcript had an open reading frame of 8535 bp that could encode a protein of 2845 amino acids if translation were initiated at the first methionine of the open

Fig. 1. Linkage between *mApc* and the Min phenotype. Genomic DNA (10 to 15 µg) isolated from parental strains and backcross animals was digested with the restriction enzyme Pst I, and Southern blot analysis was performed with *mApc*-51a as the probe. Animals with intestinal tumors were scored as Min, and tumor-free animals were scored as normal. (A) The probe identified a unique 3.4-kb fragment in the CAST DNA. This 3.4-kb fragment was present in the normal but not in the Min animals of the backcross generation. (B) The probe identified a unique 4.3-kb fragment in the AKR DNA. This 4.3-kb AKR fragment was present in the normal but not in the Min animals of the backcross generation.



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reading frame (14). The murine and human APC coding sequences were 86 and 90% identical at the nucleotide and amino acid levels, respectively. All the amino acid motifs previously identified in the human APC gene product are conserved in the *mApc* gene product. These include the heptad repeats, *ral-2* homology, muscarinic acetylcholine receptor (mAChR)-G protein coupling homology, and the 20-amino acid repeats (3, 6). A shorter, alternatively spliced transcript of *mApc*, corresponding to a previously described alternatively spliced human APC transcript

(4, 6), was also identified by RT-PCR (15).

There were 32 nucleotide polymorphisms in the coding region and 1 nucleotide polymorphism in the 3' untranslated region between B6 and CAST (14). Because all of the B6-specific polymorphisms were present in the *mApc* cDNA from the Min-affected (B6 × CAST) F₁ mouse, we concluded that the entire coding region of both alleles was expressed in Min mice.

A transversion from T to A at nucleotide 2549 (16) was found in both Min-affected mice but not in any of the three

normal mice (Fig. 2A). This Min-specific mutation converts codon 850 from a leucine (TTG) to a stop (TAG) codon. Nonsense mutations such as this one are frequently observed in FAP kindreds (5, 6). To confirm the cosegregation of the mutation with the B6 allele of the *mApc* gene, we sequenced several individual clones of RT-PCR product from the Min-affected (B6 × CAST) F₁ mouse. The results show that the nonsense mutation was present only in the B6 allele (Fig. 2B).

We confirmed the cosegregation of the nonsense mutation with the Min phenotype by allele-specific hybridization. The region of the genome spanning the mutation was amplified by PCR from 107 mice, including parental, F₁, and the backcross animals from Table 1. The PCR products were blotted onto duplicate filters and hybridized with either a mutant-specific oligonucleotide or a control oligonucleotide (Fig. 3). Only the DNA from those animals with the typical Min phenotype hybridized with the mutant-specific oligonucleotide. The discordant mouse described above did not carry the Min-specific mutation. This result is consistent with the suggestion that this animal had sporadic, rather than Min-induced, tumors (17).

Thus, a germ-line nonsense mutation in the *mApc* gene is responsible for the Min phenotype. The phenotypic and genetic similarities between murine Min and human FAP suggest that the Min mouse is an excellent animal model for the human disease FAP. Because mutation of the APC gene occurs somatically during the development of sporadic colorectal tumors in humans (5), Min mice should also be a suitable model for human colorectal cancer in

Fig. 2. Identification of a Min-specific nonsense mutation in *mApc*.

(A) A T-to-A mutation in Min mice. The mutation is indicated by an arrowhead. Cloned RT-PCR product (3, 13) derived from total RNA isolated from mice was sequenced. Lanes 1, a normal B6 mouse; lanes 2, a Min B6 mouse; lanes 3, a normal (B6 × CAST) F₁ mouse; lanes 4, a Min (B6 × CAST) F₁ mouse; and lanes 5, a normal CAST mouse. The primers used for PCR were 5'-GCCATCCCTTCACGTTAG-3' and 5'-TTCCACTTTGGCAGCATAAGGC-3', which amplified nucleotides 2241 to 2859 of the *mApc* cDNA. The primer used for sequencing was 5'-CACAAGCAGAATCTTTATGG-3', corresponding to nucleotides 2365 to 2384 of the *mApc* cDNA. (B) The cosegregation of the nonsense mutation with the B6 allele. RT-PCR product of the RNA from a Min-affected (B6 × CAST) F₁ mouse was cloned and sequenced as described (13) except that individual clones were analyzed. B6 and CAST indicate the B6 and the CAST alleles, respectively. M indicates the sequence spanning the mutated region. The mutated nucleotide is indicated with an asterisk. P indicates the sequence spanning the polymorphic region. The primers used for PCR were 5'-CAGCAGGTTATTGCAAGTG-3' and 5'-TTCCACTTTGGCATAAGGC-3', which amplified nucleotides 1615 to 2859 of *mApc* cDNA. The primer used for sequencing the mutated region was the same one used in (A). The primer used for sequencing the polymorphic region was 5'-TAGGAAACAGAAAGCTCTAG-3', corresponding to nucleotides 2256 to 2275 of *mApc* cDNA.

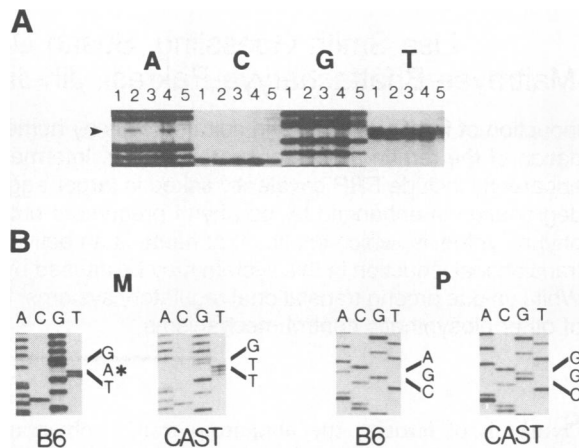


Fig. 3. Identification of the Min-specific mutation by allele-specific hybridization.

(A) Hybridization with the control oligonucleotide. (B) Hybridization with the mutant-specific oligonucleotide. (C) The pattern of samples blotted on the filters. These are PCR products unless otherwise indicated. Sources of DNA used in PCR are indicated as follows: 1, a normal AKR backcross mouse; 2, a normal AKR backcross mouse; 3, the discordant mouse described in the text; 4, a Min-affected (B6 × AKR) F₁ mouse; B6, a normal B6 mouse; AKR, a normal AKR mouse; -, a no-DNA control. Templates for cNOR and cMUT are the same cDNA from the normal and the Min-affected (B6 × CAST) F₁ mice, respectively, used in sequencing analysis. The pNOR is a plasmid subclone of normal *mApc* cDNA; pMUT is a plasmid subclone of *mApc* cDNA containing the Min-specific mutation; pBS is pBluescript SK (Stratagene, La Jolla, California). PCR reactions were performed as described (3) except that 100 to 200 ng of genomic DNA were used as template. Primers used for the PCR were the same as described in (A). Approximately 100 ng of the PCR product were blotted onto each filter. For plasmids, about 500 ng of DNA were blotted onto each filter. Hybridization was carried out as described in (9) except at 45°C and washing was carried out at 50°C in 450 mM NaCl, 18 mM sodium citrate, 1 mM tris, pH 7.2, and 0.1% SDS. The sequence for the mutant-specific oligonucleotide is 5'-ACAGAAGTTAGGAGAGAGA-3', which hybridizes to nucleotides 2540 to 2558 of *mApc* cDNA. The underlined A is the mutated nucleotide. The sequence for the control oligonucleotide is 5'-CCTCAGTGCTTACCATCC-3', which hybridizes to nucleotides 2571 to 2588 of *mApc* cDNA.

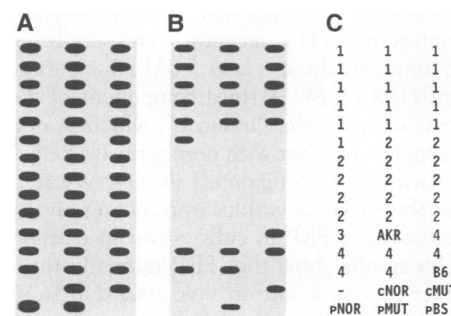


Table 1. Linkage analysis demonstrating cosegregation of the Min phenotype with *mApc*. The backcross column indicates the strain of mice crossed with the Min B6 mice to obtain the F₁ generation. Cosegregation of the Min phenotype and the B6 allele of *mApc* was determined by RFLP analysis with the *mApc*-51a probe. Animals with intestinal tumors were scored as Min, and tumor-free animals were scored as normal. The non-B6 allele is either the AKR or the CAST allele, depending on the backcross set. Animals were randomly chosen from the Min and normal set in each backcross. Seventy-four animals were analyzed from the CAST backcross set, which consisted of 135 Min and 103 normal mice. Ninety-five animals were analyzed from the AKR backcross set, which consisted of 77 Min and 77 normal mice.

Backcross	Phenotype	Alleles at <i>mApc</i>	
		B6/B6	B6/non-B6
CAST	Min	36	0
	Normal	0	38
AKR	Min	64	1
	Normal	0	30

general. Furthermore, the Min strain has permitted the identification of unlinked modifier loci in the mouse genome that decrease the number of intestinal adenomas arising in Min mice (18). Identification and characterization of potential human counterparts of these modifier loci could be useful in understanding why some FAP patients develop many fewer tumors than average (19) and in understanding colon cancer risk in the general population.

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9. Human cDNA fragments of the APC gene corresponding to nucleotides 664 to 2267 and 2517 to 3183 were used in the first round screening. Fragments 3161 to 5383 and 5762 to 7430 were used in the second round screening. Fragments -22 to 659 and 6807 to 8481 were used in the third round screening. Hybridizations were carried out at 50°C in 0.5% nonfat dried milk, 1% SDS, 6% polyethylene glycol (average molecular weight 8000), 10% formamide, salmon sperm DNA (0.2 mg/ml), 0.9 M NaCl, 0.5 mM EDTA, and 0.05 M sodium phosphate (pH 7.0). Washes were performed at 55°C in 45 mM NaCl, 1.8 mM sodium citrate, 0.1 mM tris, pH 7.2, 0.1% SDS.
10. Analysis of 22 independent clones enabled us to obtain a murine sequence corresponding to 95% of the coding region of human APC (GenBank, accession no. M74088) (3).
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12. Fifteen primer pairs for PCR and 45 internal sequencing primers were designed from the nucleotide sequence of *mApc* cDNA clones. Sequences and corresponding positions of these primers are available on request.
13. PCR products were cloned into a Bluescript vector modified [as described by T. A. Holton and M. W. Graham, *Nucleic Acids Res.* 19, 1156 (1991)]. Pools consisting of at least 100 clones were sequenced as described by J. M. Nigro *et al.* [*Nature* 342, 705 (1989)].
14. The nucleotide sequence of the B6 allele and the polymorphic changes in the CAST allele have been deposited in GenBank, accession no. M88127.
15. L.-K. Su *et al.*, unpublished data.
16. The A of the proposed translation initiation codon was designated nucleotide 1.
17. A combination of linkage analysis and allele-specific hybridization identified four additional mice from the AKR backcross that lacked the Min-specific mutation but did have intestinal tumors. Each of these animals had an increased life-span relative to most Min mice and had only 1 or 2 duodenal tumors. These results show that sporadic tumors occurred in mice from the AKR backcross.

A similar analysis of mice from the CAST backcross yielded no evidence for sporadic tumors.

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Enhanced Degradation of the Ferritin Repressor Protein During Induction of Ferritin Messenger RNA Translation

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Induction of ferritin synthesis in cultured cells by heme or iron is accompanied by degradation of the ferritin repressor protein (FRP). Intermediates in the degradative pathway apparently include FRP covalently linked in larger aggregates. The effect of iron on FRP degradation is enhanced by porphyrin precursors but is decreased by inhibitors of porphyrin synthesis, which implies that heme is an active agent. These results suggest that translational induction in this system may be caused by enhanced repressor degradation. While unique among translational regulatory systems, this process is common to a variety of other biosynthetic control mechanisms.

Synthesis of ferritin, the ubiquitous iron storage protein, is regulated at the translational level by iron in vertebrates (1, 2). The regulatory machinery consists of a conserved 28-nucleotide sequence in the 5' untranslated region of the ferritin mRNA [the iron-responsive element (IRE)] and a 98-kD protein that binds to the IRE in the absence of iron and inhibits translation. This protein is known as the ferritin repressor protein, FRP, the IRE-binding protein, or the iron regulatory factor. The FRP is a member of a family of proteins, some or all of which recognize the IRE in ferritin, transferrin (Tf) receptor, and erythroid δ -aminolevulinic acid (ALA)-synthase mRNAs (3-8). A third component of the machinery is the "inducer," which is iron complexed either with porphyrin (as heme) or with other compounds (9-11). We tested the effects of various iron sources on the turnover of FRP in cells grown in culture. Our results show that FRP normally turns over at a slow rate in vivo, but that iron, probably acting through heme, enhances the rate of FRP degradation. This effect was observed with concentrations of iron or heme that induce ferritin synthesis, which suggests that repressor degradation may be a direct cause of translational induction.

The first indication that the natural turnover of FRP might result in derepression of ferritin synthesis came from the

observation that actinomycin D causes a gradual derepression of ferritin synthesis: after 19 hours of treatment, ferritin synthesis in transformed mouse cells is derepressed to approximately half the rate achieved in the presence of iron (12). Synthesis of no other protein is increased by actinomycin D (13). Similar results have been reported with cordycepin inhibiting mRNA synthesis (11). Moreover, the maximal FRP concentration produced by treatment with the iron chelator Desferal is achieved only if concomitant protein synthesis is allowed to occur (4). These results suggest that FRP ordinarily turns over in the presence of iron, which results in derepression of ferritin synthesis and degradation of Tf receptor mRNA.

To test this hypothesis, we determined the stability of newly synthesized FRP by first labeling rabbit cells for 2 hours in the absence of an iron source and then measuring radioactivity in FRP in the presence of added iron or heme (14). The immunoprecipitable label in FRP disappears as a result of treatment with heme (Fig. 1A). An early step in this degradative pathway appears to be the covalent linking of FRP to one or more other proteins. The major linked species migrates in SDS-polyacrylamide gel electrophoresis (PAGE) at about 200 kD. These intermediates are eventually degraded, with traces of presumptive degradation products sometimes visible at about 25, 40, and 70 kD. Formation of the linked species may be reversible after a brief exposure to heme (15).

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