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Loss of *Apc*$^+$ in Intestinal Adenomas from Min Mice[1]

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ABSTRACT

Allelic loss at the *Apc* locus in spontaneously occurring intestinal adenomas from mice heterozygous for the *Apc*^{Min} nonsense mutation was analyzed using a site-specific quantitative polymerase chain reaction assay. All 97 of the intestinal adenomas analyzed showed extensive loss of the wild-type *Apc* (*Apc*$^+$) allele. Quantitative polymerase chain reaction analysis of loci linked to *Apc* indicated loss of the chromosome carrying *Apc*$^+$. Only one copy of the homologue carrying *Apc*^{Min} remained in the intestinal adenomas. Possible reasons for the difference in the mechanism of *Apc*$^+$ loss between human and Min mouse intestinal adenomas are discussed.

INTRODUCTION

Germline mutations of the *APC*[3] gene have been implicated in familial adenomatous polyposis (1–5), a dominantly inherited human syndrome in which affected individuals are predisposed to develop numerous intestinal adenomas (reviewed in Ref. 6). In addition, *APC* is somatically mutated in sporadic colorectal tumors, including small adenomas (2, 7, 8). The frequency of somatic *APC* mutations is the same in adenomas and advanced carcinomas (8). These observations suggest that mutation of *APC* is an early event in colorectal cancer.

Since the majority of *APC* alterations are inactivating mutations (2, 3, 5, 7, 8), it has been hypothesized that *APC* is a tumor suppressor gene (reviewed in Refs. 9 and 10). Because in familial adenomatous polyposis patients tumors are focal and some areas of the colon are tumor free, it can be concluded that heterozygosity for an *APC* mutation by itself is insufficient for adenoma formation. Further somatic events must be involved.

Various studies have shown detectable loss of all normal *APC* alleles or gene products in 31 to 71% of human colorectal adenomas (7, 8, 11) and 81% of colon tumor cell lines (12). These studies indicate that one mechanism for adenoma formation is the mutation of both alleles of the *APC* tumor suppressor gene.

Adenomas with no detectable mutation of both *APC* alleles could have formed by one of the following scenarios: (*a*) the mutation of both *APC* alleles occurred in the adenomas. However, only one mutation was detected because of the inadequacy of current detection methods; (*b*) an *APC* mutation in conjunction with one or more somatic genetic events at other loci would result in adenoma formation; (*c*) in a cell carrying an *APC* mutation, epigenetic events, which are somatically heritable changes that do not alter the DNA sequence, would result in adenoma formation.

Min mice are heterozygous for a nonsense mutation (*Apc*^{Min}) at the *Apc* locus, the murine homologue of *APC* (13). The Min mouse has been proposed to be a model for the study of human colorectal cancer (14). On the B6 background, Min mice develop numerous adenomas throughout the small intestine and colon. As an experimental system, Min mice have two advantages over humans: (*a*) numerous adenomas with the same inherited *Apc* mutation are available for analysis; (*b*) these adenomas develop in animals of uniform genetic background. These conditions mean that all the adenomas are equally informative and eliminate any effect of genetic heterogeneity on the sequence of events that lead to adenoma formation.

Using a site-specific quantitative PCR assay, we have analyzed intestinal adenomas from Min mice for the presence of the *Apc*$^+$ allele. To determine the mechanism of *Apc*$^+$ loss, we have analyzed intestinal adenomas from (AKR × B6-Min) F_1-*Apc*^{Min}/*Apc*$^+$ mice for the presence of SSLP alleles linked to the *Apc* locus on mouse chromosome 18 (15, 16).

MATERIALS AND METHODS

Mice. All mice were bred at the McArdle Laboratory for Cancer Research from B6 and AKR mice purchased from The Jackson Laboratory (Bar Harbor, ME). The B6-Min pedigree is maintained by crossing *Apc*^{Min}/*Apc*$^+$ males with B6 females. (AKR × B6-Min) F_1 animals were produced by mating B6-Min *Apc*^{Min}/*Apc*$^+$ males with AKR females. The *Apc*^{Min}/*Apc*$^+$ animals produced by these matings were identified by an allele-specific PCR assay described in detail previously (17).

Genomic DNA Isolation. Genomic DNA was isolated from B6, B6-Min, AKR, and (AKR × B6-Min) F_1 mice as described in detail previously (18).

Tissue Samples. Tissue samples were obtained from 12 B6-Min and 16 (AKR × B6-Min) F_1 *Apc*^{Min}/*Apc*$^+$ mice after CO_2 asphyxiation. The number of adenomas analyzed per B6-Min mouse ranged from 1 to 11 with an average of 4. For the adenomas from (AKR × B6-Min) F_1 *Apc*^{Min}/*Apc*$^+$ mice, the number analyzed from a single mouse ranged from 1 to 12 with an average of 3 adenomas. The intestinal tract was removed. The entire small intestine was divided into three sections of equal length (proximal, middle, and distal), opened longitudinally, and washed in PBS. The whole colon was opened longitudinally and washed in PBS. Tumors were dissected from each intestinal section under a dissecting microscope (at 5×). A histologically normal tissue control sample was collected from within 1 cm of each tumor.

All tissue samples were fixed overnight in PBS-buffered 10% formalin, transferred to 70% ethanol for three incubations of 24 h each, and subsequently embedded in paraffin. Paraffin sections 10 μm thick were prepared using a new microtome blade for each sample to prevent cross-contamination. Every tenth histological section was stained with hematoxylin and eosin.

The stained sections were used as templates for the isolation of tissue from the intervening unstained sections. Tissue was scraped from the slides with a new scalpel blade for each sample. The region of each section containing the least amount of histologically normal epithelial cells was collected. For each normal tissue control sample, all the histologically normal epithelium was collected.

DNA Extraction from Sections. DNA was extracted by a modification of a method described in detail previously (19). In brief, each tissue sample pooled from approximately 20 sections was placed in an Eppendorf tube and deparaffinized by two 30-min incubations with 1 ml of xylene. The xylene was removed by two washes with 1 ml of 100% ethanol. The ethanol was then removed by vacuum drying. Each sample was rehydrated with 80 μl of 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.5% Tween 20, and 200 μg of proteinase K and incubated overnight at 37°C. Undigested tissue was pelleted by centrifugation at 14,000 rpm for 5 min. The supernatant was then transferred to a new tube. Twelve μl of a slurry of Chelex 100 (Bio-Rad, Hercules, CA) in 10 mM Tris-HCl (pH 8.0)-0.1 mM EDTA were added to each sample. After vortexing,

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[3] The abbreviations used are: *APC*, adenomatous polyposis coli; B6, C57BL/6J mouse strain; AKR, AKR/J mouse strain; PCR, polymerase chain reaction; SSLP, simple sequence length polymorphism; PBS, phosphate-buffered saline, pH 7.0.

samples were incubated at 37°C for 30 min followed by boiling for 5 min. Prior to each use, samples were centrifuged at 14,000 rpm for 5 min.

PCR for the *Apc* Locus. The nucleotides of the primers MAPCHIII Forward (F) ($5'^{2524}$TCTCGTTCTGAGAAAGACAGAAGCT) and MAPCHIII Reverse (R) ($5'^{2679}$TGATACTTCTTCCAAAGCTTTGGCTAT) that differ from the *Apc* coding sequence are underlined. These base changes were necessary for the generation of *Hind*III restriction sites. Since the nucleotide alterations do not occur at the site of the Apc^{Min} point mutation [nucleotide 2549 (13)], they should not affect the amplification of the Apc^{Min} allele relative to the Apc^+ allele.

Each DNA sample (2 μl) was amplified in a 10-μl reaction containing: 0.4 μM F primer; 0.4 μM R primer; 200 μM concentrations (each) of dCTP, dGTP, dTTP, and dATP; 0.033 μM [α-^{32}P]dCTP (3000 Ci/mmol) (Dupont, Boston, MA); 2 mM $MgCl_2$; 10 mM Tris-HCl (pH 9.0 at 25°C); 50 mM KCl; 0.1% Triton X-100; and 1.0 units of Taq polymerase. All reactions were overlaid with 30 μl of mineral oil. Samples were amplified in a Coy Thermal Cycler under the following conditions: 1 cycle at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 60°C for 2 min, and 72°C for 2 min followed by 1 cycle at 72°C for 7 min and 1 cycle at 15°C for 1 min. Duplicate amplifications were done for each DNA sample.

***Hind*III Digestion.** For each *Apc* PCR product, 7 μl of amplimer were incubated at 37°C overnight in a 10-μl reaction containing 2 U *Hind*III restriction enzyme (New England BioLabs, Beverly, MA), 5 mM spermidine (Sigma Chemical Co., St. Louis, MO), 50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol (pH 7.9 at 25°C).

PCR of SSLP Markers. Two or more independent SSLP markers were jointly amplified in the same reaction tube, except for the *D18Mit20* marker which was amplified alone. The SSLP marker combinations were as follows: *D18Mit19* with *D12Mit5*; *D18Mit14* with *D7Nds1* and *D19Mit1*; *D18Mit24* with *D6Mit39*; *D18Mit33* with *D7Mit38*; and *D18Mit4* with *D3Mit51*. These SSLP markers are all polymorphic between the AKR and B6 strains (20, 21).

For each pair of SSLP markers, the reverse primers were ^{32}P end-labeled in a reaction containing 2.6 μM concentrations of each reverse primer, 0.5 μM [γ-^{32}P]ATP (6000 Ci/mmol; Dupont), 70 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 0.6 unit/μl T4 polynucleotide kinase (New England BioLabs). The reaction mixture was incubated at 37°C for 30 min followed by a 10-min incubation at 65°C to inactivate the enzyme.

For each set of SSLP markers, PCR was carried out as follows. Each DNA sample (2 μl) was amplified in a 10-μl reaction containing 0.44 μl of the end-labeling reaction product (which corresponds to a 0.114 μM concentration of each R primer); 0.114 μM of each F primer; 200 μM concentrations (each) of dCTP, dGTP, dTTP, and dATP; 2 mM $MgCl_2$; 10 mM Tris-HCl (pH 9.0 at 25°C); 50 mM KCl; 0.1% Triton X-100; and 0.25 unit of Taq polymerase. All reactions were overlaid with 30 μl of mineral oil. Samples were amplified in a Coy Thermal Cycler under the following conditions: 1 cycle at 94°C for 3 min followed by 25 cycles at 94°C for 15 s, 55°C for 2 min, and 72°C for 2 min, followed by 1 cycle at 72°C for 7 min and 1 cycle at 15°C for 1 min (21). Duplicate amplifications were done for each DNA sample.

Denaturing Gel Electrophoresis. Five μl of each *Hind*III digestion and each SSLP PCR product, except for the *D18Mit4/D3Mit51* products, were electrophoresed through 0.4-mm-thick 7.5% denaturing polyacrylamide (Bio-Rad) gels using standard conditions (23). The *D18Mit4/D3Mit51* PCR products were separated on a 6% denaturing polyacrylamide gel. In order to prevent cross-contamination of the samples during loading, alternate lanes of the gels were used.

Gels were fixed in 10% acetic acid/10% methanol for 15 min, vacuum dried at 80°C, and exposed to Kodak XAR-5 film at −70°C for at least 12 h.

Quantitation. All bands were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The background value for each band on a gel was determined by the quantitation at the same position on the gel of a lane containing an amplification reaction which had no added DNA.

For the analysis of the *Apc* locus, the ratio Apc^+/Apc^{Min} of the Apc^+ to Apc^{Min} band was calculated for each sample after correcting for background. If the Apc^+/Apc^{Min} values of the duplicate samples differed by ≤10%, they were averaged. If the values differed by more than 10%, the amplification reactions were repeated. The ratio (undigested/total) of the undigested PCR product to the sum of the radioactivity in the undigested, Apc^{Min}, and Apc^+ bands (each corrected for background) was calculated for each sample to determine the efficiency of the *Hind*III digestion. The mean undigested/total ratio was 0.02 ± 0.01. The maximum undigested/total ratio was 0.07.

For each SSLP marker, the ratio of the AKR to B6 band was calculated. The same criterion for averaging duplicate samples was utilized.

RESULTS

***Apc* Locus PCR-based Assay.** The F and R PCR primers produce a 155-base pair product from the *Apc* locus (Fig. 1). The R primer *Hind*III restriction site acts as an internal control for *Hind*III digestion of the PCR products. PCR products generated from the Apc^+ allele contain an additional *Hind*III site, generated by a deoxyadenosine at nucleotide 2549 of the Apc^+ open reading frame. A deoxythymidine is present at this position in the Apc^{Min} sequence (14). The internally [^{32}P]dCTP-labeled 155-base pair PCR product amplified from the Apc^{Min} allele is digested by *Hind*III to generate a 144-base pair product (Fig. 1*b*). The PCR product amplified from the Apc^+ allele is digested by *Hind*III to a 123-base pair fragment. Because of the difference in the number of deoxycytosine residues in the two allelic products after digestion (13), the amount of radioactivity in the Apc^+ product is 85% of the amount in the Apc^{Min} product.

Quantitation of *Apc* Locus PCR-based Assay. To determine whether the assay is quantitative, three independent amplification reactions were done for each ratio value of genomic DNA from B6-Min (Apc^{Min}/Apc^+) and B6 (Apc^+/Apc^+) mice (see Fig. 2). Since the ratio of PCR product from the two alleles compared with the allelic ratio of the genomic DNAs was linear over the range of 1.0 to 10, the PCR assay was considered to be quantitative over this range.

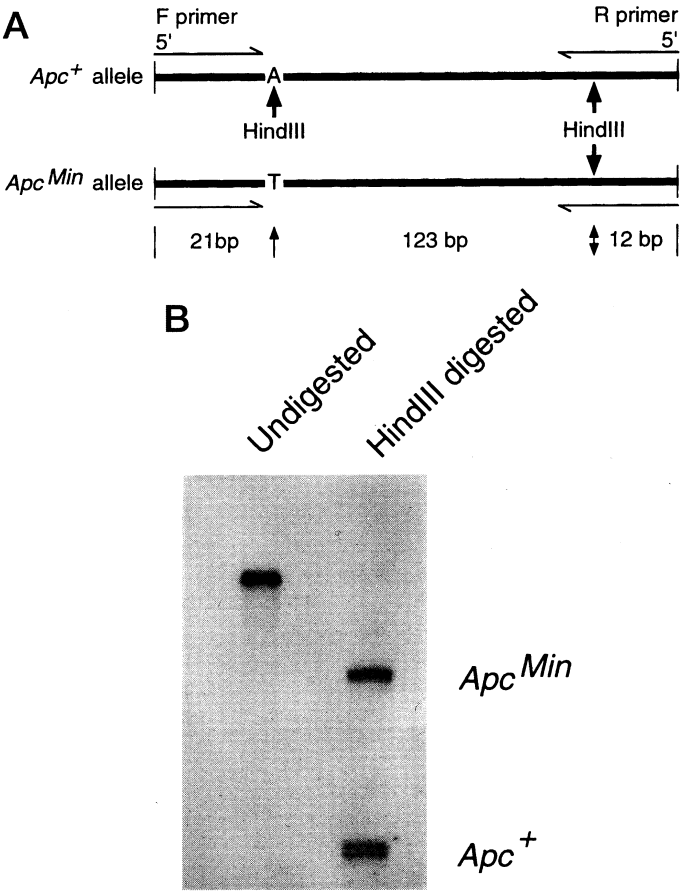

Fig. 1. *Apc* locus PCR-based assay. *A*, schematic diagram showing the difference in the *Hind*III digestion patterns for the PCR products generated from the Apc^+ and Apc^{Min} alleles. The A (deoxyadenosine) and T (deoxythymidine) nucleotides represent the single base pair (*bp*) alteration between Apc^+ and Apc^{Min} on the B6 background. *B*, an autoradiogram of a denaturing acrylamide gel showing the undigested and *Hind*III-digested PCR products generated in the assay.

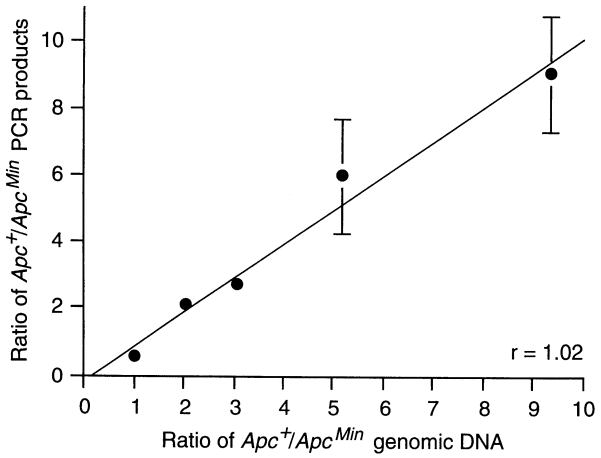

Fig. 2. Quantitation of the *Apc* locus PCR-based assay. Defined amounts of genomic DNA from B6-Min (Apc^{Min}/Apc^+) and B6 (Apc^+/Apc^+) mice were subjected to the *Apc* locus PCR-based assay. There is a linear correlation between the ratio of the genomic DNAs and the products of the PCR-based assay for the range of 1.0 to 10. *Bars,* SD. The standard deviations for the three lowest ratio values were too small to be plotted on the graph.

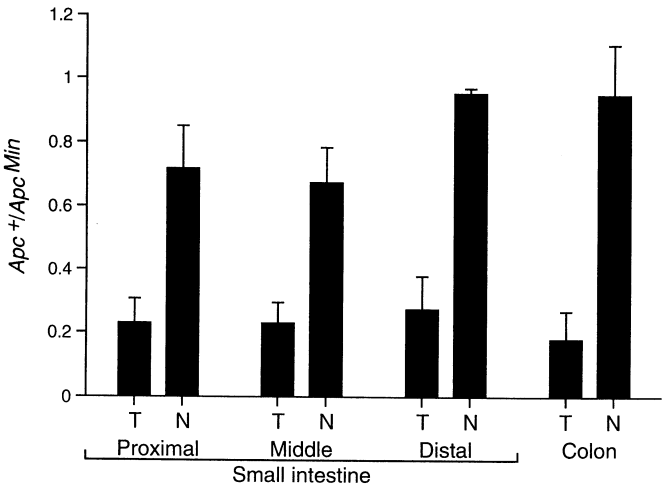

Fig. 3. Loss of Apc^+ allele in adenomas from B6-Min mice. Apc^+ to Apc^{Min} PCR product ratios generated from intestinal adenomas (*T*) and histologically normal control tissues (*N*) for the three regions (proximal, middle, and distal) of the small intestine and for the colon from B6-Min mice are shown. The allelic ratio values have not been corrected for the difference (85%) in the amount of radioactivity in the Apc^+ product relative to the Apc^{Min} product. *Columns,* means; *bars,* SD.

Analysis of Adenomas from B6-Min Mice for Apc^+ Loss. Tissues were histopathologically classified as adenomas or as normal intestinal epithelium (14). Forty-seven tumors were analyzed: 11 proximal, 10 middle, and 6 distal small intestinal adenomas; and 20 colonic adenomas. The proximal, middle, and distal portions of the small intestine roughly correspond to the duodenum, jejunum, and ileum, respectively (14). Each of the 29 normal intestinal epithelial samples originated from within 1 cm of one or more adenomas and was used as the normal tissue control. DNA was isolated from each tissue sample and analyzed for the ratio of Apc^+/Apc^{Min}, as described in "Material and Methods."

The ratio of the radioactive counts in the Apc^+ to Apc^{Min} bands was calculated for each sample (Fig. 3). The mean Apc^+/Apc^{Min} value for the adenomas was 0.21 ± 0.09 (SD) with individual ratio values not exceeding 0.37. The mean Apc^+/Apc^{Min} values for the normal tissue controls was 0.83 ± 0.18 with individual ratios values of 0.58 or greater. The difference in Apc^+/Apc^{Min} values between the adenomas and the normal tissue controls is highly significant ($P = 1.43 \times 10^{-13}$; Wilcoxon rank sum test). These results indicate that all the adenomas showed extensive loss of Apc^+.

Since the Apc^+/Apc^{Min} values for the adenomas were greater than zero, some cells in the adenoma must retain the Apc^+ allele. The fact that retention of Apc^+ is partially due to the presence of underlying normal mucosa in the adenoma sample is consistent with a comparison of colonic adenomas and adenomas of the small intestine. Colonic adenomas are frequently pedunculated and, therefore, easier to isolate free of underlying intestinal tissue than adenomas of the small intestine. Correspondingly, the Apc^+/Apc^{Min} values for the colonic adenomas (0.17 ± 0.09) are less than the values for the small intestinal adenomas (0.23 ± 0.08) ($P = 0.017$; Wilcoxon rank sum test).

Determination of the Extent of Allelic Loss on Mouse Chromosome 18. To determine if Apc^+ loss involved linked loci, a number of loci were analyzed on chromosome 18, to which *Apc* maps (15, 16). Since the only known genetic difference between B6-Apc^{Min}/Apc^+ and B6 mice is the Apc^{Min} point mutation, B6-Min adenomas are uninformative for allele loss analysis involving loci on chromosome 18 other than *Apc*. Therefore, adenomas from (AKR × B6-Min) F_1 Apc^{Min}/Apc^+ mice were analyzed. In these mice, the Apc^+ allele is carried on the AKR homologue of chromosome 18.

Fifty tumors were analyzed: 24 proximal, 17 middle, and 3 distal small intestinal adenomas; and 6 colonic adenomas. Thirty normal intestinal epithelial samples were used as the normal tissue controls. Again, each originated from within 1 cm of one or more adenomas.

DNA from each tissue sample was subjected to the *Apc* locus PCR-based assay and quantitative PCR using six chromosome 18 SSLP markers (17). The genetic map for the six chromosome 18 SSLP markers is the following: centromere-*D18Mit19*-2.4±1.7 cM-*D18Mit20*-7.1±2.8 cM-*Apc*-2.4±1.7 cM-*D1'8Mit14*-6.3±2.7 cM-*D18Mit24*-24.1±4.8 cM-*D18Mit33*-9.3±4.2 cM-*D18Mit4* (16, 17, 23).[4] These six SSLP markers cover approximately 51.6 cM of the 60-cM length of mouse chromosome 18 (23). To determine the incidence of allelic loss events for other regions of the genome, the DNA samples were amplified with six non-chromosome 18 SSLP markers. The same approach used to quantitate the *Apc* locus PCR-based assay was utilized to test the linearity of amplification for each SSLP marker used in this analysis. Since the yield of the AKR/B6 PCR product compared with the ratio of the two input DNAs for each SSLP marker was linear over the range of 0.20 to 1.0 (data not shown), the PCR assay for each SSLP marker was considered to be quantitative over this range. The slope for the SSLP marker quantitation curves ranged from 0.58 to 1.33.

Each SSLP marker used in this study does show a slight allelic bias in the amplification of the AKR and B6 alleles (see Table 1, control tissue column). For the SSLP markers *D18Mit19*, *D18Mit14*, *D18Mit24*, *D18Mit33*, *D18Mit4*, *D6Mit39*, and *D12Mit5*, there is a bias toward the amplification of the AKR allele over the B6 allele. For the remaining markers (*D18Mit20*, *D3Mit51*, *D7Nds1*, *D7Mit38*, and *D19Mit1*), the amplification bias favors the B6 allele. The amplification bias does not skew the analysis of allelic loss. The amplification bias for the chromosome 18 SSLP markers does make the ratio of the tumor to control values in Table 1 appear to increase with distance from the centromere. When each ratio is corrected for the slope of the quantitation curves, there is no correlation between the ratio value and map position (data not shown).

All 50 adenomas showed loss of Apc^+ and the AKR allele for each of the six chromosome 18 SSLP loci analyzed (Table 1). Subsets of the 50 adenomas were scored for each of the 6 non-chromosome 18 loci: *D3Mit51*, 47 adenomas, 30 control samples; *D6Mit39*, 47 ade-

[4] C. Luongo and A. R. Moser, unpublished data.

Table 1 *Allelic loss in intestinal tissue samples from (AKR × B6-Min) F_1 Apc^{Min} /Apc^+ mice*

The means ± SD for the allelic ratios at the listed loci were calculated for 50 intestinal adenomas (tumor) and 30 histologically normal intestinal epithelial samples (control tissue) from (AKR × B6-Min) F_1 Apc^{Min}/Apc^+ mice. The allelic ratio values have not been corrected for amplification bias between the B6 and AKR alleles. The chromosome 18 loci are ordered from the centromere (top, *D18Mit19*) to the telomere (*D18Mit4*). These loci span 51.6 cM of the 60-cM length of mouse chromosome 18.

Loci	Distance (cM)	*AKR/B6* Allele: Tumor	*AKR/B6* Allele: Control tissue
On chromosome 18, in map order			
D18Mit19		0.40 ± 0.13	1.46 ± 0.25
	2.4 ± 1.7		
D18Mit20		0.19 ± 0.10	0.76 ± 0.13
	7.1 ± 2.8		
Apc		0.16 ± 0.07	0.68 ± 0.16
	2.4 ± 1.7		
D18Mit14		0.35 ± 0.13	1.19 ± 0.19
	6.3 ± 2.7		
D18Mit24		0.48 ± 0.15	1.31 ± 0.20
	24.1 ± 4.8		
D18Mit33		0.43 ± 0.13	1.13 ± 0.11
	9.3 ± 4.2		
D18Mit4		0.66 ± 0.13	1.34 ± 0.12
On chromosomes other than 18			
D3Mit51		0.81 ± 0.16	0.83 ± 0.17
D6Mit39		1.56 ± 0.23	1.53 ± 0.29
D7Nds1		0.66 ± 0.11	0.62 ± 0.10
D7Mit38		0.65 ± 0.15	0.61 ± 0.20
D12Mit5		1.45 ± 0.24	1.47 ± 0.25
D19Mit1		0.77 ± 0.21	0.71 ± 0.13

nomas, 29 control samples; *D7Mit38*, 50 adenomas, 30 control samples; *D7Nds1*, 34 adenomas, 22 control samples; *D12Mit5*, 41 adenomas, 29 control samples; *D19Mit1*, 29 adenomas, 12 control samples. None of the adenomas or control samples assayed showed loss of either allele for these loci.

Determination of Chromosome 18 Copy Number. For five sets of coamplified SSLP markers (*D18Mit19/D12Mit5*, *D18Mit14/D7Nds1/D19Mit1*, *D18Mit24/D6Mit39*, *D18Mit33/D7Mit38*, and *D18Mit4/D3Mit51*) the abundance of B6 PCR product of each marker was normalized to the total amount of the non-chromosome 18 marker PCR product. The non-chromosome 18 SSLP loci could be utilized as internal normalization controls because no allelic loss was detected for any of these loci in tumors. DNA samples with replicate normalized B6 abundance values that differed by more than 0.20 were not included in this analysis. Utilizing this criterion, the replicate normalized B6 abundance values for 21 adenomas could be analyzed for 2 or more SSLP pairs. The values for 19 adenomas could be analyzed for a single SSLP pair. Since the ratio of the PCR products generated from *D18Mit14/D7Nds1/D19Mit1* coamplification varied too greatly among the replicate samples, this marker set could not be included in the analysis.

For each chromosome 18 locus, the normalized B6 abundance for each tumor was compared to its control tissue to determine the B6 allele's copy number in the adenoma (See Table 2). For each SSLP pair, the average tumor/normal tissue ratio for the *B6* allele was slightly greater than one, implying that only one copy of the B6 homologue is present in the adenoma. For each SSLP pair the normalized B6 abundance for the control tissue samples relative to one another was calculated to be approximately one.

Table 2 *Determination of copy number of the B6 homologue of chromosome 18*

SSLP pairs	Normalized B6 abundance: Tumor	Normalized B6 abundance: Control
D18Mit19/D12Mit5	1.12 ± 0.35 (18)[a]	1.15 ± 0.67 (15)
D18Mit24/D6Mit39	1.00 ± 0.27 (12)	1.04 ± 0.31 (5)
D18Mit33/D7Mit38	1.22 ± 0.28 (30)	1.04 ± 0.32 (21)
D18Mit4/D3Mit51	1.14 ± 0.44 (12)	1.16 ± 0.65 (5)

[a] Numbers in parentheses, number of samples analyzed.

DISCUSSION

One or more somatic events must occur when an intestinal adenoma forms from a cell carrying a germline *APC* mutation. An important question is the nature of these further events. Hypothetically, a somatic event could be either genetic or epigenetic. Possible genetic events include allelic loss or inactivation at *APC* or another locus or the activation of a proto-oncogene. Possible epigenetic events include DNA hypomethylation or APC polypeptide concentration fluctuations that lead to stable cellular changes (24).

Up to 71% of human colorectal adenomas and 81% of colon tumor cell lines have detectable loss of all normal *APC* alleles or protein (11, 12). These results support a two mutation hypothesis for intestinal adenoma formation where both mutations occur at the *APC* locus, in accord with Knudson's model for retinoblastoma (25).

In order to test the hypothesis that both *Apc* alleles are mutated in adenomas from Min mice, 97 intestinal adenomas [47 adenomas from B6-Min mice and 50 adenomas from (AKR ×B6-Min) F_1 Apc^{Min}/Apc^+ mice] were analyzed for the presence of the Apc^+ allele. Without exception, the adenomas showed loss of Apc^+.

However, loss of Apc^+ was not complete. Retention of the Apc^+ allele is related to the presence of underlying mucosa in the adenoma sample. An additional explanation for a small amount of Apc^+ in the adenoma samples is that the adenomas are heterogeneous at the cellular level (26). The differentiated intestinal cells which have been shown to be present in adenomas from Min mice may have retained the Apc^+ allele. Immunohistochemical analysis of adenoma samples for Apc expression would be required to determine the allelic composition of these differentiated cells.

In order to determine the mechanism for Apc^+ loss, the 50 adenomas from (AKR × B6-Min) F_1 Apc^{Min}/Apc^+ mice were analyzed for allelic loss involving six SSLP markers on chromosome 18. In these mice, the Apc^+ allele is carried on the AKR chromosome 18. The chromosome 18 markers were selected to span most of the chromosome in order to differentiate Apc^+ losses due to interstitial deletion, somatic recombination, and whole chromosome loss. Every adenoma analyzed showed loss of the AKR allele for each chromosome 18 marker. From this result, we conclude that the mechanism for Apc^+ loss is either whole chromosome loss or somatic recombination

proximal to *D18Mit19*. These mechanisms cannot be distinguished by the analysis of additional markers, since there is no known SSLP locus proximal to *D18Mit19* (17). These mechanisms can be distinguished by studying homologue copy number (see below).

None of the 50 adenomas from (AKR × B6-Min) F_1 Apc^{Min}/Apc^+ mice showed allelic loss for the six non-chromosome 18 loci. This result indicates that loss of other chromosomes in these intestinal adenomas does not appear to be a common occurrence.

In an attempt to determine the copy number of the B6 chromosome 18 remaining in the adenoma, five of the six chromosome 18 SSLP markers were co-amplified with a compatible non-chromosome 18 SSLP marker. The mean ratio (tumor/normal) was calculated for the normalized B6 abundance values. A relative abundance value of 1.0 would be expected if there was only one B6 chromosome 18 homologue in the tumor samples, because there is only one B6 chromosome 18 homologue present in the normal tissue sample (see Table 2). A value of 2.0 would be expected if there were two chromosome 18 B6 homologues present in the tumor tissue. For the coamplified markers that could be analyzed, the mean ratio (tumor/normal) for the relative abundance values is close to 1.0. From this result, it can be concluded that in the subset of tumors analyzed there is only one copy of the B6 chromosome 18. Therefore, the most likely cellular mechanism for loss of the AKR chromosome 18 is mitotic nondisjunction without reduplication.

Since only a subset of tumors could be analyzed for copy number and the range of relative abundance values is large, this result must be confirmed by an independent method for determining chromosome copy number. One possible method would be karyotyping of the adenoma samples. An alternative method, quantitative Southern blotting with independent probes for chromosome 18 and a chromosome other than 18, could be used to determine copy number for the B6 chromosome 18. Unfortunately, neither of these methods could be used on the adenomas analyzed because of the fixation method and the small size of the adenomas.

In human colorectal adenomas, both familial and sporadic, somatic genetic events involving the *APC* locus on chromosome 5q21–22 (1–4) include point mutations, small deletions, and interstitial deletions (5, 7, 8, 27–30). In no study to date has somatic loss of all of chromosome 5 been detected.

An important question raised by the results presented here is why the mechanism for the somatic mutation of the Apc^+ allele differs between the Min mouse adenomas and human intestinal adenomas. One possible reason for the difference is that hemizygosity for human chromosome 5 is not tolerated in intestinal cells. Human chromosome 5 haplotypes might always carry alleles of one or more genes that cause cell lethality in either the hemizygous or homozygous state. In contrast, inbred mouse strains, such as B6 and AKR, have been selected for homozygous viability at each locus.

A more intriguing possibility is that allelic loss of additional mouse chromosome 18 genes is needed for adenoma formation in Min mice and that haploidy for chromosome 18 is the most efficient way to bring about these multiple loss events. Of note, the mouse homologues of *MCC* (mutated in colorectal cancer) and *DCC* (deleted in colorectal carcinomas), two putative tumor suppressor genes implicated in human colorectal cancer (31, 32), map to mouse chromosome 18 (15, 16). In contrast, while *MCC* is closely linked to *APC* on human chromosome 5 (1, 4), *DCC* is unlinked [human chromosome 18 (32)]. In order to address the question of necessity of somatic loss at other loci on chromosome 18, somatic mutations that involve only the *Apc* locus must be sought in adenomas from Min mice.

In conclusion, our results show that extensive loss of the Apc^+ allele occurs in all spontaneously occurring intestinal adenomas from B6 and (AKR × B6-Min) F_1 mice heterozygous for Apc^{Min}. For the adenomas from (AKR × B6-Min) F_1 Apc^{Min}/Apc^+, the mechanism for loss of Apc^+ was shown to involve the entire chromosome 18.

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