Loss of Apc+ in Intestinal Adenomas from Min Mice

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INTRODUCTION

Germline mutations of the APC 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

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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

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Received 5/4/94; accepted 9/19/94.

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1 This work was supported by Grants ROI-CA50585, R01-23076, R01-63677, T32-GM07133, CA09135, and Core Grant CA07175 from the National Cancer Institute. This is Publication No. 3418 from the Laboratory of Genetics.

2 To whom requests for reprints should be addressed, at McArdle Laboratory for Cancer Research, 1400 University Avenue, Madison, WI 53706.

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.

(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+

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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 MAPCHIIIForward (F) (5'-5254TTCGTCTGAGGACAGACACAGCT-3') and MAPCHIIFORWARD (R) (5'-3257TGATACCTTCCAAAGACCTTGCTA-3') that differ from the Apc coding sequence are underlined. These base changes were necessary for the generation of HindIII restriction sites. Since the nucleotide alterations do not occur at the site of the Apc" point mutation [nucleotide 2549 (13)], they should not affect the amplification of 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, dATP, and dTTP; and 0.033 μg [α-32P]dCTP (3300 Ci/mmol) (Dupont, Boston, MA); 2 mM MgCl₂; 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. Each reaction was 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.

**HindIII Digestion.** For each PCR product, 7 μl of amplimer were incubated at 37°C overnight in a 10-μl reaction containing 2 U HindIII restriction enzyme (New England BioLabs, Beverly, MA); 50 mM NaCl, 10 mM Tris-HCl, 10 mM MgCl₂, and 1.0 units of Taq polymerase. All reactions were incubated at 37°C overnight in a 10-μl reaction containing 2 U HindIII restriction enzyme (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 pair of SSLP markers, the reverse primers were [32P] end-labeled in a reaction containing 2.6 μM concentrations of each reverse primer, 0.5 μM [γ-32P]ATP (6000 Ci/mmol; Dupont), 70 mM Tris-HCl (pH 7.6), 10 mM MgCl₂, 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 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 HindIII digestion and each SSLP PCR product, except for the D18Mit14/D3Mit51 products, were electrophoresed through 0.4-mm-thick 7.5% denaturing polyacrylamide (Bio-Rad) gels using standard conditions (23). The D18Mit14/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 Phosphorimagere (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⁺ of the Apc⁺ to Apc⁺ band was calculated for each sample after correcting for background. If the Apc⁺/Apc⁺ 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⁺, and Apc⁺ bands (each corrected for background) was calculated for each sample to determine the efficiency of the HindIII 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 HindIII restriction site acts as an internal control for HindIII digestion of the PCR products. PCR products generated from the Apc⁺ allele contain an additional HindIII site, generated by a deoxyadenosine at nucleotide 2549 of the Apc⁺ open reading frame. A deoxythymidine is present at this position in the Apc⁺ sequence (14). The internally [32P]dCTP-labeled 155-base pair PCR product amplified from the Apc⁺ allele is digested by HindIII to generate a 144-base pair product (Fig. 1b). The PCR product amplified from the Apc⁺ allele is digested by HindIII 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⁺⁺ 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⁺⁺/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 DNA was linear over the range of 1.0 to 10, the PCR assay was considered to be quantitative over this range.
Analysis of Adenomas from B6-Min Mice for Apc<sup>+</sup> 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 tissues were used as the normal tissue controls. Analysis of 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-D18Mit14-6.3± 2.7 cm-D18Mit24-24.1±4.8 cm-D18Mit33-9.3±4.2 cm-D18Mit4 (16, 17, 23). 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 shows 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<sup>+</sup> 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-
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 x B6-Min) F1 Apc"/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 x B6-Min) F1 Apc"/Apc" mice were analyzed for allelic loss involving six SSLP markers on chromosome 18. In these samples, the Apc" allele is carried on the AKR chromosome 18. The chromosome 18 markers were selected to span most of the chromosome 18. 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).

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Table 1 Allelic loss in intestinal tissue samples from (AKR x B6-Min) F1, Apc"/Apc" mice

<table>
<thead>
<tr>
<th>Loci</th>
<th>Distance (cM)</th>
<th>Tumor</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18Mit19</td>
<td>2.4 ± 1.7</td>
<td>0.40 ± 0.13</td>
<td>1.46 ± 0.25</td>
</tr>
<tr>
<td>D18Mit20</td>
<td>7.1 ± 2.8</td>
<td>0.19 ± 0.10</td>
<td>0.76 ± 0.13</td>
</tr>
<tr>
<td>D18Mit14</td>
<td>2.4 ± 1.7</td>
<td>0.16 ± 0.07</td>
<td>0.68 ± 0.16</td>
</tr>
<tr>
<td>D18Mit24</td>
<td>6.3 ± 2.7</td>
<td>0.35 ± 0.13</td>
<td>1.19 ± 0.19</td>
</tr>
<tr>
<td>D18Mit33</td>
<td>24.1 ± 4.8</td>
<td>0.48 ± 0.15</td>
<td>1.31 ± 0.20</td>
</tr>
<tr>
<td>D18Mit4</td>
<td>9.3 ± 4.2</td>
<td>0.43 ± 0.13</td>
<td>1.13 ± 0.11</td>
</tr>
<tr>
<td>D2Mit5</td>
<td>8.4 ± 0.6</td>
<td>0.66 ± 0.13</td>
<td>1.34 ± 0.12</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>SSLP pairs</th>
<th>Normalized B6 abundance</th>
<th>Tumor</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>D18Mit19/D12Mit5</td>
<td>1.12 ± 0.35 (18)*</td>
<td>1.15 ± 0.67 (15)</td>
<td></td>
</tr>
<tr>
<td>D18Mit24/D6Mit39</td>
<td>1.00 ± 0.27 (12)</td>
<td>1.04 ± 0.31 (5)</td>
<td></td>
</tr>
<tr>
<td>D18Mit14/D7Mit38</td>
<td>1.22 ± 0.28 (30)</td>
<td>1.04 ± 0.32 (21)</td>
<td></td>
</tr>
<tr>
<td>D18Mit24/D3Mit51</td>
<td>1.14 ± 0.44 (12)</td>
<td>1.16 ± 0.65 (5)</td>
<td></td>
</tr>
</tbody>
</table>

*a Numbers in parentheses, number of samples analyzed.
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) F1, ApcMin/+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 co-amplified 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) F1 mice heterozygous for ApcMin+. For the adenomas from (AKR × B6-Min) F1, ApcMin/+Apc+, the mechanism for loss of Apc+ was shown to involve the entire chromosome 18.

ACKNOWLEDGMENTS

We thank Karen Gould for continuing discussions and helpful critique, Norman Drinkwater for consultation on the statistical analysis, and Linda Clipson and Ilse Riegel for detailed assistance with the manuscript.

REFERENCES

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