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A resistant genetic background leading to incomplete penetrance of intestinal neoplasia and reduced loss of heterozygosity in *Apc^{Min}*/+ mice

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ABSTRACT **Previous studies of *Min*/+ (multiple intestinal neoplasia) mice on a sensitive genetic background, C57BL/6 (B6), showed that adenomas have lost heterozygosity for the germ-line *Apc^{Min}* mutation in the *Apc* (adenomatous polyposis coli) gene. We now report that on a strongly resistant genetic background, AKR/J (AKR), *Min*-induced adenoma multiplicity is reduced by about two orders of magnitude compared with that observed on the B6 background. Somatic treatment with a strong mutagen increases tumor number in AKR *Min*/+ mice in an age-dependent manner, similar to results previously reported for B6 *Min*/+ mice. Immunohistochemical analyses indicate that Apc expression is suppressed in all intestinal tumors from both untreated and treated AKR *Min*/+ mice. However, the mechanism of *Apc* inactivation in AKR *Min*/+ mice often differs from that observed for B6 *Min*/+ mice. Although loss of heterozygosity is observed in some tumors, a significant percentage of tumors showed neither loss of heterozygosity nor *Apc* truncation mutations. These results extend our understanding of the effects of genetic background on *Min*-induced tumorigenesis in several ways. First, the AKR strain carries modifiers of *Min* in addition to *Mom1*. This combination of AKR modifiers can almost completely suppress spontaneous intestinal tumorigenesis associated with the *Min* mutation. Second, even on such a highly resistant genetic background, tumor formation continues to involve an absence of Apc function. The means by which Apc function is inactivated is affected by genetic background. Possible scenarios are discussed.**

Germ-line mutation of the adenomatous polyposis coli (*APC*/*Apc*) tumor suppressor gene predisposes both humans and mice to intestinal tumorigenesis (reviewed in ref. 1). Familial adenomatous polyposis (FAP) is a dominantly inherited human cancer syndrome that results from germ-line *APC* mutation. Individuals with FAP can develop up to several thousand intestinal tumors, which are located primarily in the colon (2). The number of tumors observed in FAP individuals seems to be influenced by the site of the germ-line mutation in *APC*, environmental factors, and the effects of unlinked modifier loci (3–6).

Apc^{Min} (*Min*, multiple intestinal neoplasia) is a nonsense mutation at codon 850 of the mouse *APC* homolog that predisposes heterozygotes to intestinal tumorigenesis (7, 8). On the C57BL/6J (B6) genetic background, *Min*/+ mice average more than 70 tumors throughout the intestinal tract and rarely live beyond 150 days of age (1). Somatic *N*-ethyl-*N*-nitrosourea (ENU) treatment of B6 *Min*/+ mice indicates that intestinal tumors are preferentially initiated during the first few weeks of life (9). Tumor formation in B6 *Min*/+ mice is invariably associated with somatic loss of the wild-type *Apc* allele, apparently by chromosome loss (10). On the (AKR × B6)F_1 genetic background, *Min*/+ adenoma formation also involves *Apc*$^+$ loss (9). However, not all tumors show loss of the *Apc*$^+$ marker when the *Min* mutation is carried on certain other F_1 hybrid genetic backgrounds (11). Also, regional *Apc* deletion or nonsense mutation frequently is observed in tumors from *Min*/+ mice that have been treated somatically with γ-irradiation or ENU (11, 12).

Crosses between B6 *Min*/+ mice and the inbred strain AKR/J (AKR) led to the identification of the *Mom1* locus (13–15). *Mom1* is a semidominant modifier of intestinal tumor number in *Min*/+ mice: one AKR allele of *Mom1* (*Mom1^A*) causes a reduction in tumor number by a factor of two; two copies of *Mom1^A* reduce tumor multiplicity by a factor of four (16). Analysis of *Min*/+ mice carrying a *Pla2 g2a* transgene indicates that this secreted phospholipase comprises at least a portion of *Mom1* (17).

Beyond *Mom1*, the AKR strain carries alleles of other loci that modify intestinal tumor number in *Min*/+ mice (14). In this paper we have examined the full extent to which the intestinal phenotype of *Min*/+ mice can be modified by transferring the *Apc^{Min}* mutation from the sensitive B6 strain onto AKR. We have found that *Min*-induced intestinal tumorigenesis is almost completely suppressed on the homozygous AKR background. We have observed that a significant percentage of tumors from AKR *Min*/+ mice do not show *Apc*$^+$ allele loss. Comparing these observations with previous analyses of the (B6 × AKR)F_1 genetic background (10), we conclude that the reduced frequency of *Apc*$^+$ allele loss of AKR is recessive to the high frequency conferred by B6. In this paper, we have asked further whether tumor formation on this highly resistant background requires functional inactivation of both *Apc* alleles. Immunohistochemical analysis has revealed that *Apc* gene expression is greatly reduced in all of the tumors that retain the *Apc*$^+$ allelic marker. Several very different scenarios can account for this observation.

Abbreviations: *APC*/*Apc*, adenomatous polyposis coli gene of the human/mouse; B6, C57BL/6J; ENU, *N*-ethyl-*N*-nitrosourea; Het_{Apc}, *Apc* heterozygosity index; LOH, loss of heterozygosity; *Min*, multiple intestinal neoplasia; *Mom1*, modifier of Min 1; *Mom1^A*, AKR allele of *Mom1*.

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MATERIALS AND METHODS

Mice. All mice were bred at the McArdle Laboratory for Cancer Research. Parental AKR mice were purchased from The Jackson Laboratory. All of the AKR *Min*/+ mice used in these experiments had been backcrossed to AKR for 5–9 generations. Thus, these AKR *Min*/+ mice were homozygous for AKR alleles at approximately 97% of all loci. The tumor multiplicity of these mice is comparable to that of mice that are now at the 11th backcross generation (data not shown). Animals were genotyped for the presence of the Apc^{Min} mutation by a previously described PCR-based assay (14). For mutagenesis studies, all mice from a litter (consisting of AKR *Min*/+ and +/+ mice) were given an i.p. injection of ENU from a single sample as described previously (9). To control further for ENU variability, litters from each age group were included in each round of ENU treatment. Litters of 10- to 15-day-old B6 *Min*/+ mice also were included in each round of ENU treatment. Tumor multiplicity in these control mice was comparable to results previously published (9).

Tumor Scoring and Collection. Intestinal tumors were scored at ×10–30 magnification as previously described (9). For experiments requiring unfixed tissue, tumor and normal intestinal tissues were carefully dissected, with a fresh scalpel blade for each sample. All other samples were fixed overnight in 10% buffered formalin or, for immunohistochemistry, for 1 hr in periodate-lysine-paraformaldehyde (18).

Quantitative PCR Analysis of *Apc*. *Apc* allelic status was assessed by a quantitative PCR assay described previously (10, 12). This assay generates 123-bp and 144-bp products from the Apc^{+} and Apc^{Min} alleles, respectively. The Apc^{+}/Apc^{Min} ratio was determined by quantitation of ^{32}P-labeled products (10). To be consistent with previously reported results, the allelic ratios were not corrected for the difference in the sizes of the Apc^{Min} and Apc^{+} amplimers (10, 12). All samples were independently amplified by PCR at least twice. Only samples that gave repeatable values (within 10%) with signals at least 10-fold above background were included in the results.

Examination of *Apc* for Truncation Mutations. Codons 680-1230 of the *Apc* ORF were examined for truncation mutations with an *in vitro* synthesized protein assay as described previously (12). This region also includes the germ-line *Min* nonsense mutation, which served as a positive control for the assay.

Immunohistochemical Analysis of Apc Expression. Tissue samples used for immunohistochemistry were fixed for 1 hr in periodate-lysine-paraformaldehyde. Antibody staining was performed essentially as described by Merritt *et al.* (19). Affinity-purified rabbit polyclonal antibody APC2, raised against amino acids 1035–2130 of human APC (a gift from Paul Polakis, Onyx Pharmaceuticals, Richmond, CA), was used at a dilution of 1:100 (20). Rabbit polyclonal antibody 3122, raised against amino acids 8–347 of human APC, was used at a dilution of 1:500 (21). The regions of APC used to generate the APC2 and 3122 antibodies are more than 87% identical to mouse Apc at the amino acid level (8). Slides were lightly counterstained with hematoxylin before dehydration and coverslipping. Staining of each sample always was accompanied by a negative control sample in which the primary antibody was omitted. No staining was observed in any of these control samples (data not shown).

RESULTS

Intestinal Tumor Multiplicity in AKR *Min*/+ Mice. When counting intestinal tumors in B6 *Min*/+ mice, we typically score four regions representing approximately 40% of the intestine. These regions consist of the entire colon and 4-cm segments from the proximal, middle, and distal small intestine. We consistently have found that B6 *Min*/+ mice scored in this manner develop 30 ± 10 tumors (1). To examine further the effects of genetic background on *Min*-dependent intestinal tumorigenesis, we introduced the *Min* mutation onto the AKR background (see *Materials and Methods*).

A summary of the intestinal tumor incidence and multiplicity for AKR *Min*/+ mice is shown in Table 1. Owing to the low tumor multiplicity of AKR *Min*/+ mice, the entire length of the small and large intestine was scored for tumors in these animals. Only 43 of 170 AKR *Min*/+ mice developed even a single intestinal tumor. In total, 53 intestinal tumors were identified from this set of 170 AKR *Min*/+ mice, yielding a multiplicity of 0.3 tumors per mouse. All of these tumors were located in the small intestine. Correcting for the difference in length of intestine scored, the tumor multiplicity of AKR *Min*/+ mice represents a reduction by approximately two orders of magnitude from that observed in B6 *Min*/+ mice (Table 1). Only one intestinal tumor was found in a total of 37 AKR $Apc^{+/+}$ mice.

The modifier effects of the *Mom1* locus previously were studied by using a strain in which the AKR allele of *Mom1* had been introgressed onto the B6 background (15). B6 $Mom1^{A/A}$ *Min*/+ mice averaged 7.8 ± 3.4 intestinal tumors, and the proportion of *Min*/+ mice with tumors remained 100% (Table 1) (15). Thus, *Mom1* can account for only a small portion of the reduction in tumor multiplicity between B6 *Min*/+ and AKR *Min*/+ mice.

Stimulation of Intestinal Adenoma Formation in AKR *Min*/+ Mice by Treatment with ENU. We previously have shown that ENU treatment of B6 *Min*/+ mice at 5–14 days of age increased tumor multiplicity approximately 3.8-fold, whereas treatment between 20 and 35 days resulted in only a 1.6-fold increase (9). These results suggest that intestinal tumors in B6 *Min*/+ mice are preferentially initiated during the first few weeks of life. To investigate the timing of tumor induction in AKR *Min*/+ mice, we injected *Min*/+ and +/+ mice with a single dose of ENU at either 9–16 or 27–42 days of age. Mice were sacrificed approximately 150 days after treatment, and tumors were scored along the entire length of the small and large intestine.

ENU treatment significantly increased both the incidence and multiplicity of tumors for each treatment group relative to untreated AKR *Min*/+ mice ($P < 0.001$ for comparisons of tumor number, Wilcoxon rank sum test, Table 2). However, a much stronger effect was seen for mice treated at 9–16 days of age. One hundred percent of these mice developed intestinal tumors, with an average multiplicity of 12.8. By contrast, 79% of AKR *Min*/+ mice treated at 27–42 days of age developed

Table 1. Comparison of intestinal tumor multiplicity in AKR and B6 *Min*/+ mice

Mice*	Number of mice with tumors	Tumor multiplicity, average ± SD	Average age, days
B6 *Min*/+	68/68 (100%)	29.2 ± 9.4	85
B6 $Mom1^{A/A}$ *Min*/+	21/21 (100%)	7.8 ± 3.4	120
AKR *Min*/+	43/170 (25%)	0.3 ± 0.5†	138‡

For AKR *Min*/+ mice, the entire length of the small and large intestine was scored for tumors. For B6 *Min*/+ and B6 $Mom1^{A/A}$ *Min*/+ mice, four representative regions comprising approximately 40% of the entire intestinal tract were scored. The entire intestinal trace was also scored for a total of 37 AKR $Apc^{+/+}$ mice (average age of 182 days). One tumor was found in the small intestine of one of these mice. No tumors were found in the entire intestinal tract of 25 B6 $Apc^{+/+}$ mice (average age of 99 days).

*Data for the B6 *Min*/+ and B6 $Moml^{A/A}$ *Min*/+ mice were taken from refs. 9 and 15, respectively.

†In total, 53 intestinal tumors were found in 170 AKR *Min*/+ mice. All tumors were in the small intestine.

‡This lifespan is limited by the predisposition of AKR to lymphomagenesis.

Table 2. Intestinal tumor multiplicity in ENU-treated AKR *Min*/+ mice

Age at ENU treatment	Number of *Min*/+ mice with tumors	Tumor multiplicity, average ± SD
9–16 days	18/18 (100%)	12.8 ± 5.9
27–42 days	19/24 (79%)	1.9 ± 1.6
Untreated	9/23 (39%)	0.5 ± 0.8

Entire litters were treated with ENU as described in *Materials and Methods*. The entire length of the small and large intestine was scored. The average age (post-ENU) for the ENU-treated mice was 156 days. The average age for the untreated mice was 152 days. No tumors were found in any of 39 ENU-treated AKR $Apc^{+/+}$ mice, 12 of which were treated at 9–16 days of age and the remaining 27 mice at 27–42 days of age. The average post-ENU age of the $Apc^{+/+}$ mice was 138 days.

tumors, with an average multiplicity of only 1.9 (Table 2). These average tumor numbers represent, respectively, 25.6- and 3.8-fold increases over untreated mice. Note that no intestinal tumors were found in any of the 39 ENU-treated AKR $Apc^{+/+}$ mice. These results indicate that, as observed for B6 *Min*/+ mice, intestinal tumors in AKR *Min*/+ mice are preferentially initiated before weaning. Colonic tumorigenesis was strongly suppressed even in ENU-treated AKR *Min*/+ mice. Only one colonic tumor was found, in an AKR *Min*/+ mouse treated with ENU at 16 days of age.

Analysis of *Apc* Allelic Status in Intestinal Tumors from AKR *Min*/+ Mice. All spontaneous intestinal tumors examined from B6 *Min*/+ and (AKR × B6)F_1 *Min*/+ mice show loss of the Apc^+ allele, apparently resulting from chromosome loss (10). This pathway for inactivating tumor suppressor function is designated loss of heterozygosity (LOH). Because there were major differences in intestinal tumor multiplicity between B6 *Min*/+ and AKR *Min*/+ mice, we investigated whether there are also differences in the somatic events associated with tumor formation.

The allelic status of the *Apc* locus was examined by a quantitative PCR assay of DNA from 16 intestinal tumors from untreated AKR *Min*/+ mice. Tumors from the AKR *Min*/+ mice tend to be quite small; however, the amount of stromal cell infiltration seemed comparable to that observed for tumors from B6 *Min*/+ mice. To obtain samples enriched for normal or tumor cells, we prepared DNA from cells that were scraped from fixed and sectioned tissue samples. The index of heterozygosity at the *Apc* locus, Het_{Apc}, was defined as the Apc^+/Apc^{Min} band ratio of the tumor DNA sample, normalized to the band ratio determined in parallel for the DNA sample from adjacent normal tissue (see Fig. 1). If the Het_{Apc} was ≥0.64, we concluded that the Apc^+ allelic marker persisted. By this criterion, 37% (6/16) of the fixed tumors from untreated AKR *Min*/+ mice maintained heterozygosity for the *Apc* marker (Fig. 1).

Tumors from AKR *Min*/+ mice that maintained the Apc^+ allelic marker might have acquired mutations within the Apc^+ allele. We previously have found that at least 27% (25/91) of intestinal tumors from ENU-treated B6 *Min*/+ mice that fail to show LOH instead carry a detectable somatic *Apc* truncation mutation (12). To begin to address this possibility for the AKR *Min*/+ case, we analyzed tumors from both untreated and ENU-treated mice for *Apc* truncation mutations, using the *in vitro* synthesized protein (IVSP) assay. In this initial analysis we focused on the region of *Apc* between codons 680 and 1230, because all of the 25 truncation mutations found in tumors from ENU-treated B6 *Min*/+ mice were found in that region of the gene. A set of frozen tissue samples was collected for these experiments, because the IVSP assay is incompatible with nucleic acids prepared from fixed tissue.

Frozen tumors from untreated and ENU-treated AKR *Min*/+ mice were analyzed. Twenty tumors (2/6 and 18/36, respectively) maintained *Apc* heterozygosity (Fig. 1). Of these,

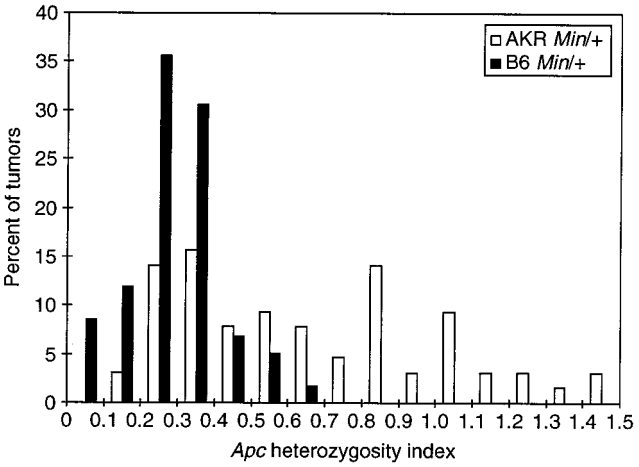

FIG. 1. *Apc* heterozygosity index for adenomas in B6 *Min*/+ and AKR *Min*/+ animals. The *Apc* heterozygosity index, Het_{Apc}, was defined by the Apc^+/Apc^{Min} band intensity ratio for tumor DNA, normalized to that ratio for DNA from adjacent normal tissue. For 59 tumors arising spontaneously in B6 *Min*/+ animals (filled bars), the values of Het_{Apc} are distributed approximately as Gaussian with mean 0.32 and SD 0.17. For 64 tumors (22 spontaneous and 42 arising after ENU treatment) in AKR *Min*/+ animals (empty bars), the distribution of Het_{Apc} differs strongly from that of B6 *Min*/+ tumors ($P = 3.5 \times 10^{-9}$ by the Wilcoxon rank sum test). The AKR *Min*/+ indices are well approximated by a mixture of two Gaussian distributions, with 53% of the tumors centered around 0.32 (the LOH class) and 47% of the tumors around 0.95 as determined by the method of maximum likelihood. With a definition of maintenance of heterozygosity as $Het_{Apc} \geq 0.64$, the probability of misclassifying a tumor with LOH is only 3%. No significant differences in the distribution of Het_{Apc} for tumors from AKR *Min*/+ mice were observed as a function of ENU treatment or method of tumor dissection and DNA isolation.

two untreated and 10 ENU-treated tumors were successfully examined by the *in vitro* synthesized protein assay. In contrast to ENU-treated B6 *Min*/+ mice, where 25/91 of the tumors that maintained the Apc^+ marker showed truncations (9), none of the 12 tumors in AKR *Min*/+ mice that maintained the Apc^+ marker showed truncations. The truncation product produced by the *Min* allele was observed in all samples, serving as a positive control for the assay. In the absence of evidence for LOH at the *Apc* locus or for truncation mutations in the Apc^+ allele, is Apc^+ expressed in tumors from the AKR *Min*/+ stock?

Analysis of Apc Expression in Intestinal Tumors from AKR *Min*/+ Mice. To explore the Apc expression in intestinal tumors from AKR *Min*/+ mice more thoroughly, we examined the Apc polypeptide by immunohistochemistry, using the amino-terminal 3122 and central APC2 polyclonal antibodies (see *Materials and Methods*).

Cytoplasmic Apc staining was observed in cells from normal intestinal epithelia of B6 *Min*/+ and AKR *Min*/+ mice (Fig. 2). Apc staining was more intense for cells in the upper half of crypts than at the base. The columnar epithelial cells of the intestinal villi showed light, but consistent, Apc staining (Fig. 2). Both 3122 and APC2 gave similar results, and this pattern of expression is consistent with previous reports of APC/Apc expression (18, 22–24). However, there were some differences between 3122 and APC2 with respect to intracellular localization of Apc. Each antibody detected Apc expression in the basal, lateral, and apical cytoplasm (Fig. 2). However, 3122 stained the apical cytoplasm much more intensely than the basal cytoplasm, whereas APC2 strongly stained both the basal and the apical cytoplasm (compare Fig. 2 *B* and *F*).

In intestinal tumors from B6 *Min*/+ mice, Apc expression was essentially undetectable with either antibody (Fig. 2 *A* and *B*). Because the 3122 antibody should be capable of recogniz-

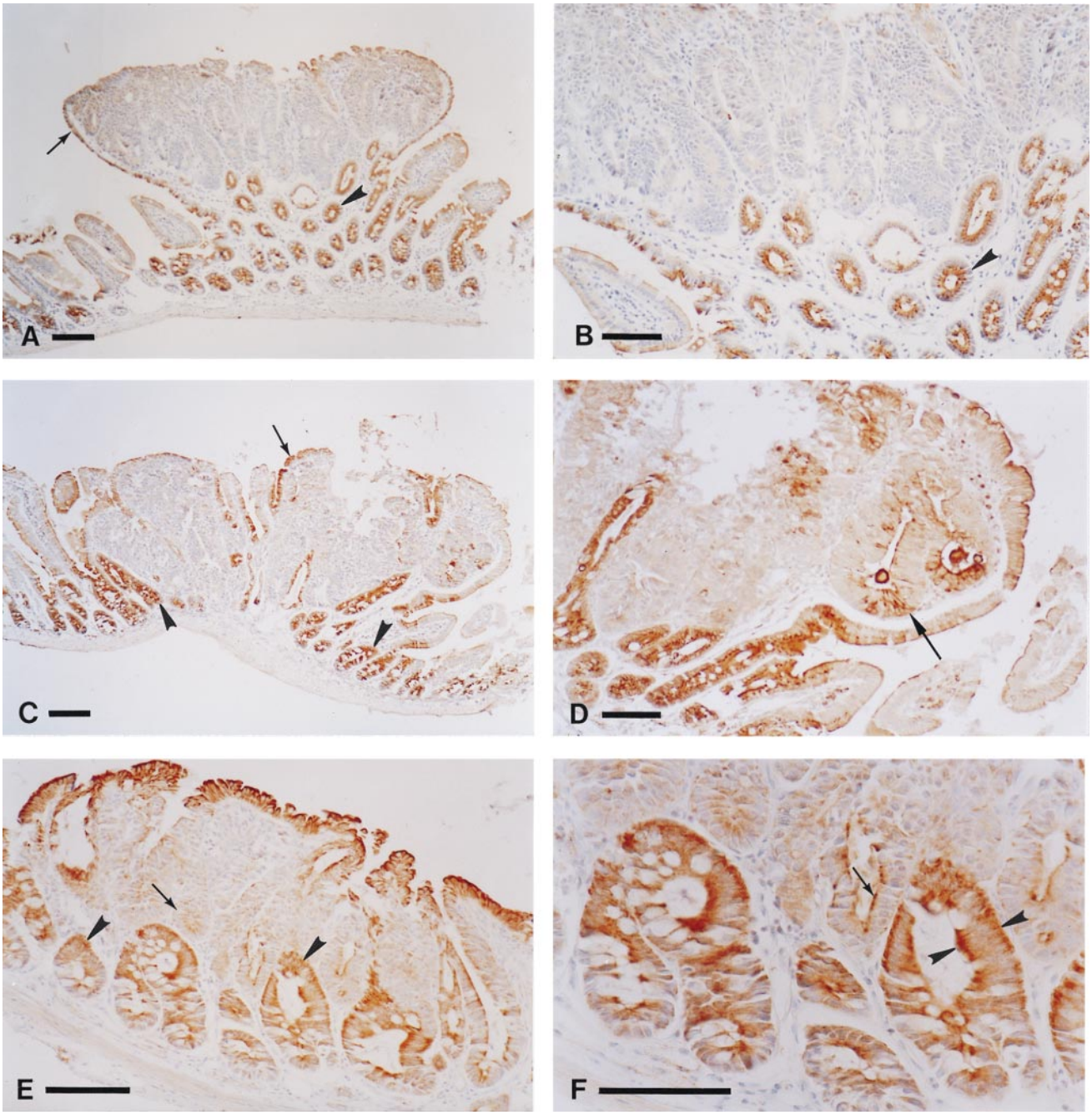

FIG. 2. Analysis of *Apc* expression in tumors from the small intestine of *Min*/+ mice. Periodate-lysine-paraformaldehyde-fixed tissues were stained with anti-Apc antibodies as described in *Materials and Methods*. The tumors in *A*, *B*, *E*, and *F* were classified by quantitative PCR as showing LOH, whereas the tumor in *C* and *D* maintained heterozygosity at *Apc*. A tumor from an untreated B6 *Min*/+ mouse stained with the 3122 antibody is shown at low (*A*) and high (*B*) magnification. Apc staining is observed in normal crypts (*A*, arrowhead) and in the normal cells that encapsulate the tumor (*A*, arrow). Note the strong Apc staining in the apical cytoplasm in the cells from the normal crypts (*B*, arrowhead). A tumor from an ENU-treated AKR *Min*/+ mouse stained with 3122 is shown in *C* and *D*. Apc staining is seen in normal crypts (*C*, arrowheads) and in the cells that encapsulate the tumor (*C*, arrow). A different, serial section of a region of the tumor in *C* is shown at higher magnification in *D*. Low levels of staining are observed in some of the tumor cells (*D*, arrow). A tumor from an untreated AKR *Min*/+ mouse stained with the APC2 antibody is shown at low (*E*) and high (*F*) magnification. Apc staining is much stronger in the cells of the normal crypts (*E*, arrowheads) relative to the tumor cells (*E*, arrow). Staining also is seen in the normal cells that encapsulate the tumor. Note the apical and strong basal cytoplasmic staining in the normal crypts (*F*, arrowheads, compare with *B*). Some residual staining can be seen in the apical cytoplasm of some of the tumor cells (*F*, arrow). (Bars, 100 μm for *A*, *C*, and *E*; 75 μm for *B*, *D*, and *F*.)

ing the Apc^{Min} polypeptide, the lack of 3122 staining in tumors from B6 *Min*/+ mice was surprising. Indeed, Western blot analysis, with a mAb specific for amino acids 1–29 of APC, detects the polypeptide fragment from the Apc^{Min} allele in colonic tumors from B6 *Min*/+ mice (1). Therefore, the native conformation of the Apc^{Min} fragment polypeptide may be unrecognizable by the 3122 antibody or the fragment may be expressed at levels not detectable by immunohistochemistry. Altogether, immunohistochemistry was used to examine 18 tumors from the small intestine of both untreated (nine tumors) and ENU-treated (nine tumors) AKR *Min*/+ mice (Fig. 2 *C–F*). For three of the 18 samples, the APC2 signal was too light to be reliably interpreted in normal and tumor cells. Twelve of the 15 scorable tumors also were analyzed by

quantitative PCR: eight maintained heterozygosity for the *Min* marker (two untreated and six ENU-treated). In the cells of all scorable samples, Apc expression detected by immunohistochemistry was significantly reduced relative to staining levels in adjacent normal cells (Fig. 2 *C–F*). However, some residual Apc staining was observed in all but two of the AKR *Min*/+ tumors. This residual staining was not found in the most dysplastic regions of the tumors. Because this residual signal was observed even in tumors classified as involving LOH, it may be nonspecific.

Taken together, these results indicate that normal Apc function(s) must be attenuated for both spontaneous and ENU-induced intestinal tumor formation in AKR *Min*/+ mice. However, the mechanism(s) of *Apc* inactivation in tumors of AKR *Min*/+ mice frequently differs from that observed for B6 *Min*/+ mice.

DISCUSSION

The Strong Dependence of a Mouse Cancer Model on Genetic Background. Manipulation of the genetic background alone can reduce intestinal tumor multiplicity in mice carrying the Apc^{Min} mutation by about two orders of magnitude (Table 1). Indeed, 75% of *Min*/+ mice on the AKR genetic background were tumor free at 6 months of age. This finding contrasts sharply with the B6 background, where 100% of *Min*/+ animals develop numerous intestinal tumors by 2–3 months of age (7). Furthermore, from the entire set of 170 AKR *Min*/+ mice examined, only one colonic tumor was identified—in an ENU-treated animal. These results highlight the importance of evaluating genetic background when studying the effects of any germ-line mutation in the mouse. To appreciate fully the phenotype(s) associated with a gene, it seems imperative to study each germ-line mutation for that gene on several different genetic backgrounds.

Even on the highly resistant AKR genetic background, intestinal tumors in *Min*/+ mice appear to initiate preferentially early in life. A 26-fold increase in tumor number was observed for mice treated with ENU at 9–16 days, whereas mice treated at 27–42 days showed only a 3.8-fold increase in tumor number compared with the untreated set. The enhanced susceptibility of younger B6 and AKR *Min*/+ mice to tumor formation may be influenced by developmental changes that occur in the intestine during the first few weeks of life. Intestinal crypt purification and expansion are two changes that may be important contributors to this susceptibility. The effect of the AKR genetic background on tumor multiplicity in *Min*/+ mice does not seem to involve alteration of the age dependence of tumorigenesis.

The mechanism of somatic *Apc* inactivation also was influenced by the genetic background. In contrast to the 100% LOH observed for untreated B6 mice, 36% (8/22) of the tumors from untreated AKR *Min*/+ mice failed to show LOH by our assay. Also, in our previous analysis of ENU-treated B6 *Min*/+ mice, 25/91 tumors lacking LOH had shown truncations, all between codons 700 and 1215 (12). No such truncation mutations in *Apc* were observed in 12 tumors from AKR *Min*/+ mice. Consider the null hypothesis that the AKR pattern does not differ from the B6 pattern. On this hypothesis, the probability that no truncations would be found in this sample is 0.036 (Fisher exact test). Thus ENU treatment on the AKR background either alters the *Apc* mutational spectrum relative to B6 or stimulates tumorigenesis by influencing loci other than *Apc*. Mutational activation of β-catenin would be one possibility for this latter scenario, but note that, in contrast to the reported case (25), the *Apc* locus always is silenced in the cases we have studied.

There are numerous intriguing possibilities raised by these results. First, it is reasonable to expect that tumor multiplicity would be influenced by factors that affect the rate or frequency of somatic *Apc* segregation. It is worth noting that even a small change in mitotic fidelity could have a large effect on tumor number in a tissue as mitotically active as the intestinal epithelium. The possibility of differences between the B6 and AKR strains in modifier loci that influence mitotic segregation requires further investigation.

At least in respect to the *Apc* locus and mouse chromosome 18, we classify B6 as mitotically unstable and AKR as relatively more stable. The instability generated by B6 is dominant to the stability of AKR, because adenomas in $(AKR \times B6)F_1$ animals show a 100% incidence of LOH (10). These germ-line differences in frequency of LOH are interesting to compare with recent observations on cell lines derived from human colon cancer. Lengauer and his colleagues (26) have described two classes of cell lines from human colonic tumors, one class karyotypically unstable and the other karyotypically stable. They have suggested that the normal stem cell precursor for colonic tumors is karyotypically stable (27). Cell fusion analysis indicates that karyotypic instability is at least partially dominant to stability (26). One cause adduced for dominant karyotypic instability is mutation of the mitotic checkpoint gene, *Bub1* (28). Several lines of further investigation are required to ascertain whether the difference in LOH frequency between the AKR and B6 backgrounds reflects generalized karyotypic instability, a difference in stem cell source for tumors (29), or germ-line differences at mitotic checkpoint loci such as *Bub1*.

Mom1 is the first mapped polymorphic modifier locus that affects intestinal tumor number in *Min*/+ mice. However, *Mom1* cannot fully account for the reduced tumor multiplicity observed in AKR *Min*/+ mice (Table 1). Homozygosity for the AKR-resistance allele of *Mom1* reduces tumor multiplicity by a factor of four relative to B6 *Min*/+ mice (16). Thus, tumor number is reduced by an additional factor of 60 by making the *Min* mutation congenic on the AKR background. Clearly, the AKR strain carries alleles at loci other than *Mom1* that strongly influence *Min*-induced tumorigenesis.

***Apc* as a Canonical Tumor Suppressor Locus.** The canonical genetic pathway for inactivating a "tumor suppressor" function involves two hits, one affecting each allele of an autosomal locus (30). When this pathway is inefficient, other mechanisms can come into play. We identified 32 tumors from AKR *Min*/+ mice in which *Apc* did not appear to be somatically inactivated either by allele loss or by truncation mutation (Fig. 1 and above). The Apc-negative status of this set of tumors cannot be readily explained by missense mutations in the wild-type allele. Missense substitutions should not simultaneously affect the multiple epitopes detected by the 3122 and APC2 antibodies. But note that the stimulation of adenoma formation on the AKR background by ENU is observed only in Apc^{Min}/+ heterozygotes. Thus, a two-hit hypothesis remains tenable: a germ-line lesion in *Apc* combined with a somatic mutation in an interacting locus. The initial two-hit hypothesis of Knudson (30) did not restrict the hits to the two alleles of one locus. The complexities of the genetics of Wilms' tumor have been interpreted by such two-locus models (31).

Although the primary mechanism of *Apc* inactivation may differ between B6 and AKR *Min*/+ mice, all intestinal tumors examined from both untreated and ENU-treated AKR *Min*/+ mice showed a dramatic deficiency in Apc expression relative to normal cells (Fig. 2). We can only speculate at this stage as to how the Apc^{+} allele becomes silent in the apparent absence of LOH or somatic mutation (32). This result demonstrates that lack of Apc tumor suppressor function is critical for tumor formation in both strains. These findings support the hypothesis of *APC*/*Apc* as a unique "gatekeeper" gene that functions as a critical negative regulator mediating intestinal epithelial homeostasis (33). Whether all neoplastic pathways are negatively regulated by a unique "gatekeeper" is an interesting issue for the future.

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