[CANCER RESEARCH 55, 4479–4485, October 1, 1995]

N-Ethyl-*N*-nitrosourea Treatment of Multiple Intestinal Neoplasia (*Min*) Mice: Age-related Effects on the Formation of Intestinal Adenomas, Cystic Crypts, and Epidermoid Cysts[1]

Alex R. Shoemaker, Amy R. Moser,[2] and William F. Dove[3]

McArdle Laboratory for Cancer Research [A. R. S., A. R. M., W. F. D.] and Laboratory of Genetics [A. R. S., W. F. D.], University of Wisconsin Medical School, Madison, Wisconsin 53706

ABSTRACT

The timing of intestinal tumor initiation in B6-*Min*/+ mice has been examined by treating mice at 5–35 days of age with a single i.p. injection of the direct-acting alkylating agent *N*-ethyl-*N*-nitrosourea (ENU). Treatment of *Min*/+ mice at 5–14 days of age resulted in a 3.8-fold increase in intestinal tumor multiplicity over untreated mice. Mice treated at 20–35 days of age showed only a 1.6-fold increase in tumor number. These results, in conjunction with examination of tumor multiplicities of untreated *Min*/+ mice as a function of age, suggest that the majority of intestinal tumors in *Min*/+ mice are initiated relatively early in life. *Min*/+ mice treated with ENU also showed an increase in the number of cystic intestinal crypts. However, the relationship between age at ENU treatment and cystic crypt multiplicity was distinct from that seen for intestinal adenomas. Mice treated at 5–9 days of age showed only a 1.9-fold increase in cystic crypts over untreated animals. By contrast, the increase in average cystic crypt multiplicity for mice treated at 10–35 days of age was 4.5-fold. In addition, 60% of *Min*/+ mice treated with ENU before 25 days of age developed epidermoid cysts, an extracolonic manifestation commonly associated with familial adenomatous polyposis in humans.

INTRODUCTION

The ability of many carcinogens to induce tumors in mice is strongly influenced by various factors including the sex (1), strain (1–3), and treatment age of the animal (1, 3, 4). Perinatal treatment of animals with alkylating agents such as ENU[4] often results in the formation of tumors in the lungs (1, 3, 5, 6), liver (1, 5, 6), lymphoid organs (1, 5, 6), and nervous system (1, 7). ENU does not commonly induce intestinal tumors in most strains of mice. However, Oomen *et al.* (5) have shown that *H-2* haplotype can strongly influence intestinal tumor incidence in mice treated with ENU at 15 days of age.

We have been investigating intestinal neoplasia in mice that are heterozygous for Apc^{Min} (*Min*), a mutant allele of the mouse homologue of the human *APC* gene (8). On the C57BL/6J (B6) background, *Min* is a fully penetrant, autosomal dominant mutation that predisposes mice to develop multiple intestinal adenomas (9). B6-*Min*/+ mice develop approximately 50 adenomas throughout the intestinal tract and rarely live beyond 150 days of age (9). Analysis of longer-lived hybrid mice carrying the *Min* mutation has shown that the majority of intestinal adenomas are established by 100 days of age (10).

In this report, we have more rigorously examined the timing of intestinal adenoma initiation in *Min*/+ mice. Specifically, we have examined whether there is a correlation between age at treatment with ENU and intestinal tumor multiplicity in *Min*/+ mice. The results presented here suggest that intestinal adenomas in *Min*/+ mice are most likely to be initiated during the first several weeks of life. In addition, we have examined the relationship between age at ENU treatment and the multiplicity of a distinct type of intestinal lesion, the cystic crypt. We have also found a relationship between age at ENU treatment of *Min*/+ mice and the development of epidermoid cysts of the skin.

MATERIALS AND METHODS

Mice. B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at the McArdle Laboratory for Cancer Research. The B6-Min pedigree is maintained by crossing B6-+/+ females with B6-*Min*/+ males (9). All B6-Min mice used in these experiments were from the twenty-sixth to thirty-first backcross generations to B6. Animals were genotyped using DNA isolated from blood samples collected from the retroorbital sinus shortly after weaning (approximately 40 days of age) or from spleen tissue collected at the time of sacrifice (11). *Min*/+ animals were identified using an allele-specific PCR assay described previously (11).

ENU Treatments. Mice from entire B6-+/+ X B6-*Min*/+ litters were given single i.p. injections of the alkylating agent ENU (Sigma Chemical Co., St. Louis, MO) at a dose of 50 μg/gm body weight. All injections were performed as described previously (12). All animals were 5 to 35 days of age at the time of ENU treatment. To analyze the results more effectively, the mice were grouped into sets of 5–9, 10–14, 15–19, 20–25, and 30–35 days of age, based on the age at the time of treatment. The weighted average treatment ages for these groups were: 6.7, 11.7, 16.4, 22.6, and 32.6 days, respectively. Litters from at least four of these five age groups were included in each round of treatments to control for any ENU variability. Each age group included 16–25 *Min*/+ mice. Wild-type littermates served as +/+ treated controls. Six ENU-treated mice, one female and five males, died before the end of the experiment; therefore, they could not be analyzed. Three of the males were determined to be *Min*/+; the other three animals were not genotyped before death. These six animals came from all treatment age groups except for the 5–9-day group.

Tumor Scoring. Animals were killed when moribund or 65 days after injection. Untreated animals that corresponded in age to the final ages of treated animals were also examined. Approximately one-third of the small intestine from each mouse was scored for tumors by examining 4-cm sections from the proximal, middle, and distal small intestine. The entire large intestine was also scored for tumors (9). For these experiments, tumors were counted under a dissecting microscope at ×10 after the intestine had been spread out flat on bibulous paper (Baxter Scientific Products, McGraw Park, IL), fixed overnight in 10% buffered formalin, and subsequently washed in 70% ethanol. Cystic crypts were quantitated concurrently from the same intestinal regions at ×10–40. All tumor and cystic crypt counts were performed by a single observer (A. R. S.). Tumors and cystic crypts from ENU-treated *Min*/+ and +/+ animals were analyzed histologically as described previously (9).

Statistical Analyses. The Wilcoxon rank sum test was used for all comparisons of total intestinal tumor or cystic crypt multiplicity of *Min*/+ mice (13). $P < 0.05$ was considered to indicate a significant difference between sample populations. This nonparametric statistical test was used for these analyses because it requires few assumptions about the distribution of the tumor or cystic crypt multiplicity data (13). Comparisons of the regional distribution of tumors and/or cystic crypts along the length of the intestinal

Received 5/10/95; accepted 7/28/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grants R01CA50585, Program-Project Grant CA23074, and Core Grant CA07175 from the National Cancer Institute. This is Publication No. 3446 from the Laboratory of Genetics.

[2] Present address: Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792.

[3] To whom requests for reprints should be addressed, at McArdle Laboratory for Cancer Research, University of Wisconsin Medical School, 1400 University Avenue, Madison, WI 53706.

[4] The abbreviations used are: ENU, *N*-ethyl-*N*-nitrosourea; Min, multiple intestinal neoplasia; APC, adenomatous polyposis coli.

tract between two sample populations were performed using the χ^2 test based on 4 x 2 contingency tables constructed by calculating the percentage of total tumor or cystic crypt multiplicity in the proximal, middle, and distal small intestine and the large intestine (14). $P < 0.05$ was used to reject the null hypothesis that the regional distribution of tumors and/or cystic crypts along the length of the intestine between two sample populations was not different. This test was used because it permitted simultaneous comparisons of all four intestinal regions between sample populations (14). The intestinal tumor incidence of +/+ mice treated with ENU at different ages was compared using Student's t test (14). The t test was also used to compare the incidence of epidermoid cysts in *Min*/+ mice treated with ENU at different ages. $P < 0.05$ was considered to indicate a significant difference in tumor incidence between sample populations.

RESULTS

Intestinal Tumors in ENU-treated *Min*/+ Mice

To determine if there is a relationship between age at ENU treatment and intestinal adenoma multiplicity, tumor numbers were compared between groups of mice treated with ENU at 5 to 35 days of age. All mice were examined for intestinal tumors at approximately 65 days after ENU treatment. The intestinal tumor multiplicities for *Min*/+ mice treated with a single injection of ENU at various ages are shown in Fig. 1*A*.

Intestinal tumor counts were also done on 68 untreated *Min*/+ mice that ranged from 67 to 97 days of age. Statistical analysis revealed no difference in tumor multiplicities for mice within this age range (Wilcoxon rank sum test; $P \geq 0.07$ for comparisons between groups of untreated animals that were 67–74, 75–80, 81–84, 85–90, or 95–97 days of age). Therefore, the untreated animals were pooled for comparisons to the treated animals (Fig. 1*A*).

All mice treated with ENU had significantly higher average tumor numbers in comparison to untreated animals ($P \leq 1 \times 10^{-6}$ for each comparison of the ENU-treated groups to the untreated control animals). However, tumor multiplicity was also dramatically affected by the age at time of treatment. The average tumor multiplicity for *Min*/+ mice treated at 5 to 9 days of age (104.1) was not different from that for animals treated at 10 to 14 days of age (116.2; $P = 0.24$; Fig. 1*A*). However, animals treated at 15 to 19 days of age had an average of 74.1 tumors. This was significantly different from the tumor multiplicity for mice treated before 15 days of age ($P < 8 \times 10^{-6}$). Tumor multiplicity continued to decline for mice treated at older ages. Mice treated at 20–25 days of age averaged 54.3 intestinal tumors, while the average for treatment at 30 to 35 days of age was 41.4 tumors (Fig. 1*A*). Each of these decreases represents a highly significant change from the average tumor multiplicity for animals treated at the next youngest age group ($P \leq 0.01$ for each comparison between successive ages of treatment).

In summary, treatment of *Min*/+ mice at 5–14 days of age resulted in a 3.8-fold increase in average tumor multiplicity. After 14 days of age, each increase of 5 days in treatment age resulted in a significant decrease in tumor multiplicity. For mice treated at 30–35 days of age, the average tumor multiplicity was only 1.4-fold higher than the average tumor number for untreated animals.

The regional distribution of tumors along the length of the intestinal tract was examined by calculating the percentage of total tumor multiplicity in each of the four counted regions of the intestine for untreated mice and each of the ENU-treated groups of *Min*/+ mice (Fig. 2). The regional distribution of tumors in treated mice closely paralleled that seen in untreated mice (Fig. 2). Comparisons of the tumor distribution in untreated *Min*/+ animals to each of the five ENU treatment groups revealed no difference in regional tumor distribution (Fig. 2; χ^2 test; $P > 0.10$). ENU treatment at different ages also did not affect greatly the regional tumor distribution (Fig. 2). The only possibly significant difference in regional tumor distribution between age groups was for treatment at 5–9 days of age *versus* treatment at 20–25 days of age (Fig. 2; $P < 0.03$). The weighted percentage of total tumor multiplicity (based on the number of ani-

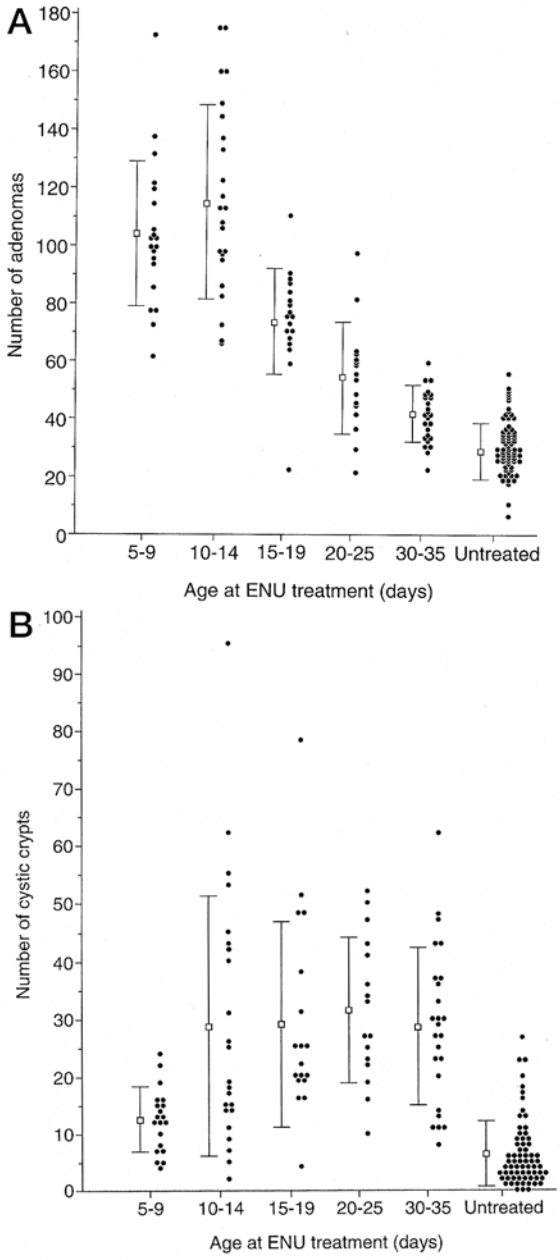

Fig. 1. *A*, intestinal tumors in ENU-treated *Min*/+ mice. ●, the number of intestinal tumors for individual *Min*/+ mice. The number of *Min*/+ mice examined for the 5–9-, 10–14-, 15–19-, 20–25-, and 30–35-day age groups was 20, 23, 18, 16, and 25, respectively. □ with *vertical bars*, the means and SDs for the indicated age groups, respectively. The numerical values for the means and SDs for each age group are as follows: 5–9 days, 104.1 ± 25.1; 10–14 days, 116.2 ± 33.0; 15–19 days, 74.1 ± 17.8; 20–25 days, 54.3 ± 18.7; 30–35 days, 41.4 ± 9.0; and untreated (67–97 days of age), 29.2 ± 9.4. ENU treatment at all ages examined led to a significant increase in average tumor multiplicity relative to untreated animals ($P \leq 1 \times 10^{-6}$ for each comparison of ENU-treated groups to the untreated control animals). However, the most significant increase was seen in animals treated at 5 to 14 days of age ($P < 1 \times 10^{-11}$). *B*, cystic crypts in ENU-treated *Min*/+ mice. ●, the number of cystic crypts for individual *Min*/+ mice. □ with *vertical bars*, the means and SDs for the indicated age groups, respectively. The numerical values for the means and SDs for each age group are as follows: 5–9 days, 12.5 ± 5.5; 10–14 days, 28.8 ± 22.6; 15–19 days, 29.2 ± 17.5; 20–25 days, 31.6 ± 12.5; 30–35 days, 28.7 ± 13.6; and untreated (67–97 days of age), 6.4 ± 6.0. ENU treatment at all ages examined led to significant increases in average cystic crypt multiplicity relative to untreated animals ($P \leq 3 \times 10^{-5}$ for each comparison of ENU-treated groups to the untreated control animals).

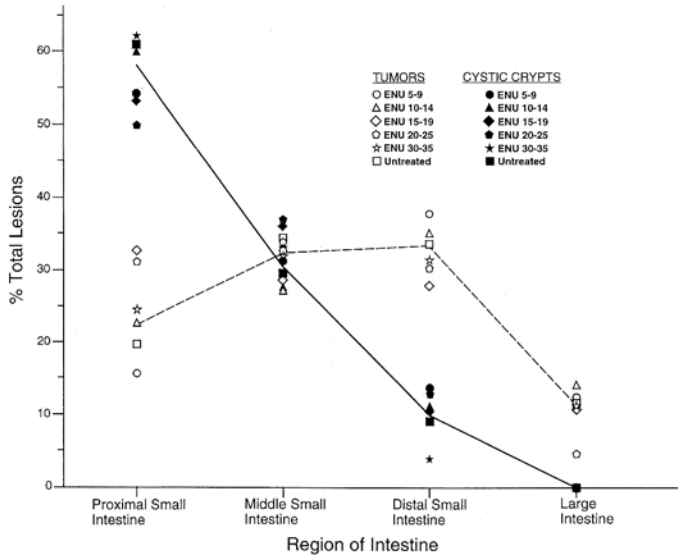

Fig. 2. Regional distribution of tumors and cystic crypts along the length of the intestinal tract in untreated and ENU-treated *Min*/+ mice. The percentage of total tumor multiplicity for untreated and ENU-treated *Min*/+ mice in each of the four intestinal regions is indicated by *open symbols*. The percentage of total tumor multiplicity in each of the four regions of the intestine for untreated (67–97 days of age), ENU-treated at 5–9 days, 10–14 days, 15–19 days, 20–25 days, and 30–35 days, respectively, are as follows. Proximal small intestine: 19.9, 16.2, 23.3, 32.5, 31.3, and 24.6%. Middle small intestine: 34.6, 32.9, 27.6, 28.7, 34.1, and 31.6%. Distal small intestine: 33.6, 38.7, 35.1, 27.8, 30.2, and 32.1%. Large intestine: 12.0, 12.2, 14.0, 10.9, 4.4, and 11.6%. ---, the weighted percentage (based on the number of animals in each group) of total tumor multiplicity for combined untreated and ENU-treated animals. The regional distribution of tumors in ENU-treated *Min*/+ mice was not different from untreated mice ($P > 0.10$). The only two ENU treatment groups that showed a significant difference in regional tumor distribution from each other were the 5–9- and 20–25-day groups ($P < 0.03$). The percentage of total cystic crypt multiplicity in each of the four regions of the intestine is indicated by *filled symbols*. The percentage of total cystic crypt multiplicity in each of the four regions of the intestine for untreated (67–97 days of age), ENU-treated at 5–9 days, 10–14 days, 15–19 days, 20–25 days, and 30–35 days, respectively, are as follows. Proximal small intestine: 60.9, 54.4, 60.1, 53.4, 50.3, and 62.4%. Middle small intestine: 29.7, 31.2, 27.8, 36.0, 36.4, and 33.4%. Distal small intestine: 9.4, 14.4, 12.2, 10.6, 13.3, and 4.2%. No cystic crypts were seen in the large intestine of any mice. ——, the weighted percentage (based on number of animals) of total cystic crypt multiplicity for combined untreated and ENU-treated animals. The regional distribution of cystic crypts was not different between untreated and ENU-treated animals ($P > 0.50$) or between groups of animals treated with ENU at different ages ($P > 0.10$). In all cases, the regional distribution of cystic crypts along the length of the intestine was significantly different from the regional distribution of intestinal tumors ($P < 0.005$).

mals in each group) in each of the four intestinal regions for combined untreated and ENU-treated mice was as follows: proximal small intestine, 23.0%; middle small intestine, 32.3%; distal small intestine, 33.3%; and large intestine, 11.4% (Fig. 2).

There was no correlation between the sex of the animal and the total tumor multiplicity for any age group (Wilcoxon rank sum test; $P \geq 0.10$ for each comparison between sexes for treatment groups). Tumors collected from ENU-treated *Min*/+ mice were not histologically distinguishable from tumors in untreated *Min*/+ mice (data not shown).

Intestinal Tumors in ENU-treated +/+ Mice

ENU-treated animals that typed as +/+ for the *Apc* locus were also scored for intestinal tumors. Mice that had tumors in any of the four regions of the intestine described above were subsequently examined for additional tumors in the remaining portions of the intestine. Seventeen percent (8 of 48) of the +/+ mice injected with ENU at 5 to 14 days of age developed tumors by 65 days after treatment (Table 1). In comparison, only 4% (3 of 75) of the +/+ mice treated at 15 to 35 days of age developed intestinal tumors (Table 1). ENU treatment of +/+ mice at 5 to 14 days of age was, therefore, significantly more likely to induce intestinal tumors than treatment at 15 to 35 days of age (Student's t test, $P < 0.005$). The total number of tumors for ENU-treated +/+ animals ranged from one to three tumors with a mean of 1.5. One of the tumors was in the large intestine, while the rest were in the proximal part of the small intestine. Excluding the single tumors seen in the 15–19- and 30–35-day age groups, tumors from each age group of +/+ mice were examined histologically. These tumors resembled typical intestinal adenomas in *Min*/+ mice (data not shown). No intestinal tumors were found in any untreated +/+ mice (Table 1).

Cystic Lesions in ENU-treated *Min*/+ Mice

Cystic Crypts. Cystic crypts were counted at the same time and in the same intestinal regions as were intestinal tumors. These lesions are morphologically and histologically distinct from intestinal adenomas (Fig. 3). Serial sectioning of intestinal samples revealed these structures to be crypts that appear to be sealed off and lined with abnormal cells that are often anaplastic (Fig. 3*b*). The cysts were typically covered by a layer of histologically normal epithelium. Cystic crypts were primarily located in the upper one-third of the small intestine of both untreated and ENU-treated *Min*/+ mice. No cystic crypts were identified in the large intestine of any mice. The cysts ranged in diameter from ~0.1 mm to approximately the size of a small adenoma (~0.50 mm).

There was no difference in cystic crypt multiplicity for untreated *Min*/+ mice that ranged in age from 67 to 97 days of age (Wilcoxon rank sum test; $P \geq 0.24$ for comparisons between groups of untreated animals that were 67–74, 75–80, 81–84, 85–90, and 95–97 days of age). For this reason, cystic crypt counts for all untreated animals were pooled for comparisons to the treated animals (Fig. 1*B*). Of 68 untreated *Min*/+ animals that were 67–97 days of age, 65 (96%) had cystic crypts. The average number of cysts for these 68 animals was 6.4 (Fig. 1*B*).

The relationship between age at ENU treatment and the number of intestinal cystic crypts is presented in Fig. 1*B*. Treatment with ENU at all ages led to significant increases in the number of cystic crypts compared to untreated *Min*/+ mice ($P \leq 3.2 \times 10^{-5}$ for each comparison of ENU-treated groups to the untreated animals). Mice treated with ENU at 5 to 9 days of age averaged 12.5 cystic crypts. Treatment at all other ages resulted in significant increases in the number of cystic crypts relative to the 5–9-day group ($P \leq 5.7 \times 10^{-5}$ for comparisons of the 10–14-, 15–19-, 20–25-, and 30–35-day treatment groups to the 5–9-day treatment group). However, there was no difference in average cystic crypt multiplicity for mice treated at 10–14, 15–19, 20–25, or 30–35 days of age ($P \geq 0.45$ for comparisons between these age groups). The average number of cysts for all *Min*/+ mice treated with ENU at 10 to 35 days of age was 29.4 ± 17.0.

As with the comparisons of regional intestinal tumor distribution,

Table 1 *Intestinal tumors in ENU-treated +/+ mice*

The number of +/+ animals with intestinal tumors approximately 65 days after treatment with ENU at the indicated ages is shown. Seventeen % of animals treated at 5 to 14 days of age developed tumors in comparison to only 4% of mice treated at 15 to 35 days of age. The total number of intestinal tumors for ENU-treated +/+ mice ranged from 1–3, with an average of 1.5 tumors per animal.

Age at ENU treatment (days)	No. of +/+ mice	No. of mice with intestinal tumors
5–9	21	3 (14%)
10–14	27	5 (19%)
15–19	18	1 (6%)
20–25	32	1 (3%)
30–35	25	1 (4%)
Untreated	25	0

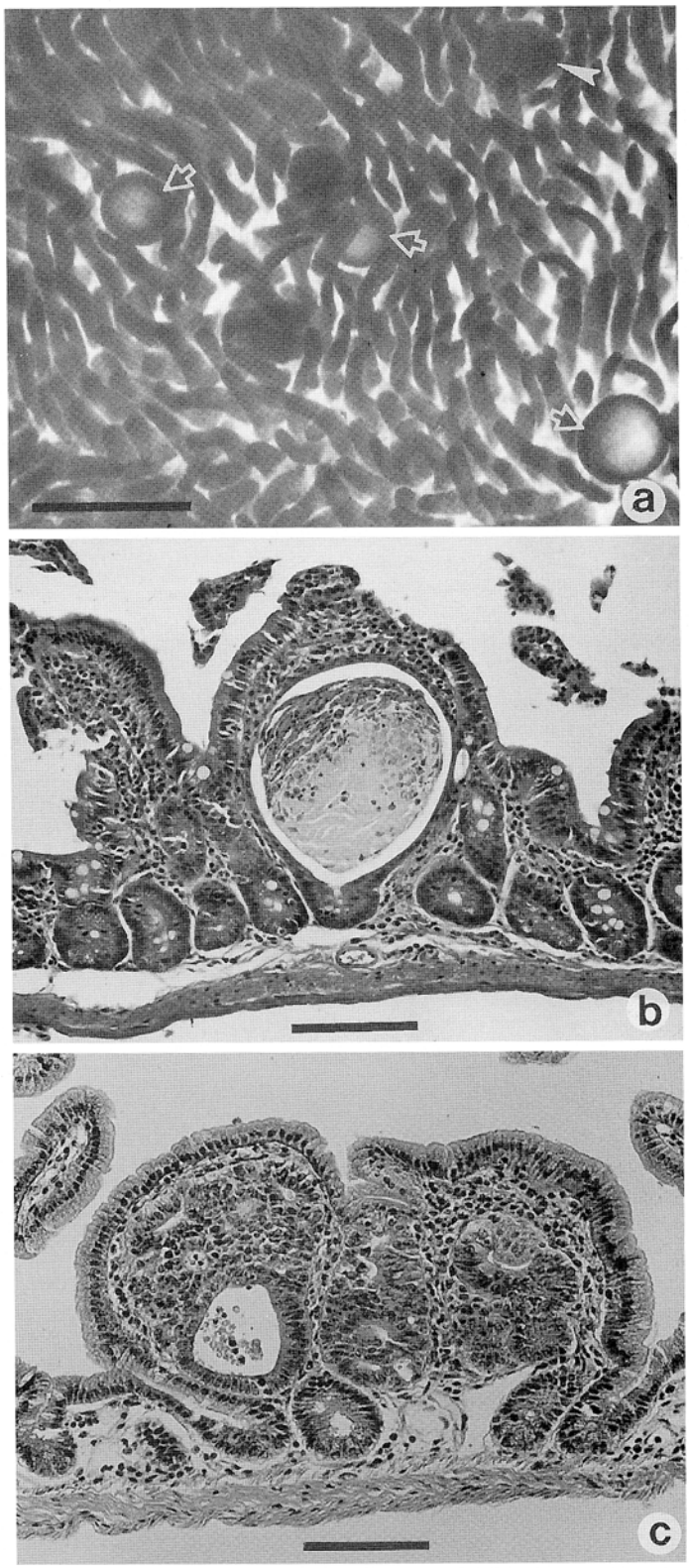

Fig. 3. *a*, cystic crypts and adenomas in the small intestine of an ENU-treated *Min*/+ mouse. A photograph of the small intestine of a *Min*/+ mouse treated with ENU at 17 days of age is shown. *Arrows*, several cystic crypts; *arrowhead*, an adenoma. *Bar*, 1.0 mm. *b*, photograph of a H&E-stained section of a cystic crypt from a *Min*/+ mouse treated with ENU at 30 days of age. The jejunal crypt shown in this figure is sealed off and lined with abnormal cells; it is filled with what appears to be cellular debris. *Bar*, 0.1 mm. *c*, photograph of a H&E-stained section of an intestinal adenoma from the small intestine of a *Min*/+ mouse treated with ENU at 12 days of age. This adenoma is comparable in size to the largest observed cystic crypts. *Bar*, 0.1 mm.

the regional distribution of cystic crypts along the length of the intestinal tract was examined by calculating the percentage of total cystic crypt multiplicity in each of the four counted regions of the intestine for untreated groups and each of the ENU-treated groups of *Min*/+ mice (Fig. 2). ENU treatment did not alter the regional distribution of cystic crypts along the length of the intestinal tract (Fig. 2; χ^2 test, $P > 0.50$ for each comparison of untreated animals to the ENU-treated groups). There was also no difference in the regional distribution of cystic crypts between groups of animals treated with ENU at different ages ($P > 0.10$). The weighted percentage of total cystic crypt multiplicity (based on the number of animals in each group) in each of the four intestinal regions for combined untreated and ENU-treated mice was as follows: proximal small intestine, 58.7%; middle small intestine, 31.1%; distal small intestine, 10.2%; and large intestine, 0% (Fig. 2). No cystic crypts were seen in either untreated or ENU-treated +/+ mice.

The regional distribution of cystic crypts along the length of the intestinal tract was clearly distinct from that seen for intestinal tumors. Comparisons of regional cystic crypt distribution in each of the untreated or ENU-treated groups with the regional tumor distribution in each of these groups revealed a significant difference in each case ($P < 0.005$; Fig. 2).

Epidermoid Cysts. Epidermoid cysts were first noted in the third set of animals to be treated with ENU. For this reason, mice from the first two rounds of mutagenesis are not included in these analyses. All 126 animals (83 *Min*/+) from subsequent rounds of ENU treatment were scored for the presence or absence of these lesions at the time of sacrifice.

Min/+ mice treated with ENU had a high incidence of epidermoid cyst development. These pigmented lesions were primarily located in the skin of the back and were often found in association with hair follicles in the subdermal layer of the skin (Fig. 4). Epidermoid cysts were found in mice of both sexes and at all treatment ages. ENU treatment on or before 25 days of age resulted in 60% (40 of 67) of *Min*/+ mice developing epidermoid cysts. Only 6% (1 of 16) of *Min*/+ mice treated at 30–35 days of age developed these lesions. *Min*/+ mice were, therefore, significantly more likely to develop epidermoid cysts when treated with ENU on or before 25 days of age (Student's *t* test, $P < 0.005$). The total number of visible cysts per animal varied from one to several dozen. No epidermoid cysts were seen in 36 untreated *Min*/+ mice that were 70–97 days of age. Of the 43 +/+ mice treated with ENU at 5 to 35 days of age, one mouse treated at 25 days of age developed a single, visible epidermoid cyst. This cyst was histologically similar to lesions seen in *Min*/+ mice (data not shown).

DISCUSSION

We have demonstrated previously that there is no difference in average intestinal tumor multiplicity for (AKR/J × B6)F_1 *Min*/+ mice ranging from 100–300 days of age (10). This result led us to examine the timing of intestinal tumor initiation in *Min*/+ mice. Here we have described experiments that determined the age-related sensitivity of intestinal tumor initiation by the chemical carcinogen ethylnitrosourea. ENU is a direct-acting, short-lived alkylating agent with a half-life of less than 1 h under the conditions used in these experiments (15). Treatment of *Min*/+ mice with a single i.p. injection of this carcinogen at 5 to 14 days of age increased intestinal tumor multiplicity 3.8-fold relative to untreated mice (Fig. 1*A*). ENU treatment at 20 to 35 days of age resulted in only a 1.6-fold increase in intestinal tumors. The short life span of *Min*/+ mice on the B6 background precluded examination of the effect of treating mice with ENU at older ages. These results demonstrate that ENU-induced

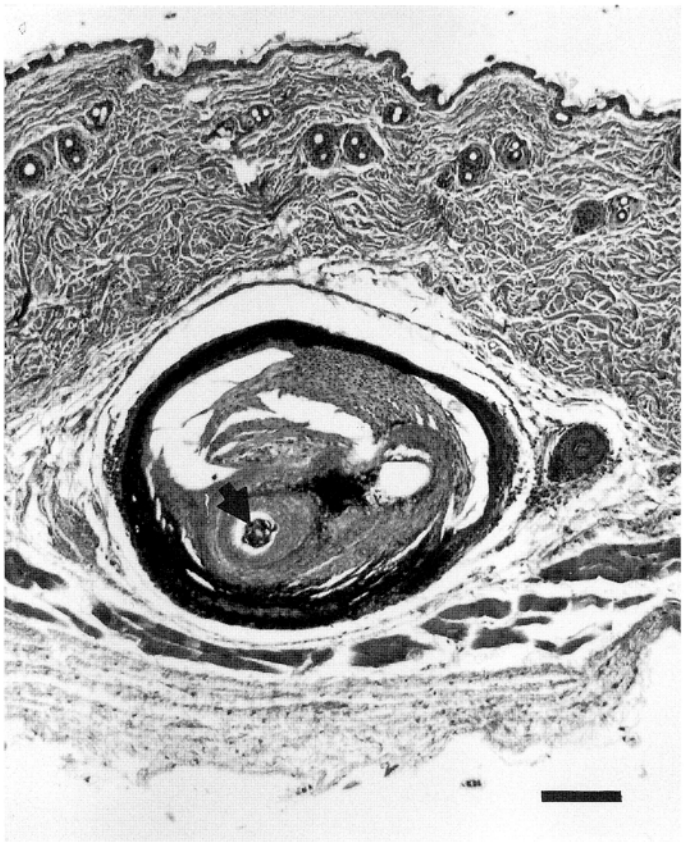

Fig. 4. H&E-stained section of an epidermoid cyst from a *Min*/+ mouse treated with ENU at 5 days of age. The cyst was located in the subdermal layer of the skin, and the cross-section of a hair follicle can be seen near the center of the lesion (*arrow*). *Bar*, 0.1 mm.

intestinal tumors in *Min*/+ mice are most likely to be initiated during the first 2 weeks of life. In addition, the tumor multiplicity of untreated *Min*/+ mice remained unchanged in the interval from 67 to 97 days of age. Mice that did not carry the *Min* mutation were also more susceptible to intestinal tumor development when treated at 5–14 days of age (Table 1). These results suggest that tumors arising spontaneously in *Min*/+ mice are also initiated relatively early in life and that this period of enhanced tumor initiation susceptibility is not unique to mice carrying the *Min* mutation. Our findings are in agreement with those of Laird *et al.* (16), which suggest that inhibition of polyp initiation in *Min*/+ mice by treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine is most effective when administered before 50 days of age.

The results presented here must be interpreted in the context of what is known about postnatal development of the intestine in the mouse. The intestinal epithelium of both mice and humans consists of a single layer of cells organized structurally into crypts, which are surrounded by villi in the small intestine. It is believed that intestinal tumors originate from stem cells located near the base of each crypt (17). Ponder *et al.* (18) have shown that, in mice, individual adult crypts are clonal structures. However, intestinal crypts in the mouse actually begin development as polyclonal structures, and clonality is not established until approximately day 14 of postnatal development through a process known as crypt purification (19). Therefore, during the first 2 weeks of life, there may be a larger population of target cells/crypt that could acquire a tumor-initiating mutation.

In addition, the number of intestinal crypts increases by as much as 77% during the third week of life (20–22). The mechanism for this increase seems to involve a process of crypt fission whereby a preexisting crypt splits into two (22–23). A mutation acquired before crypt fission could, therefore, be inherited by multiple crypts.

Developmental changes in stem cell mitotic index and/or cell cycle time could influence the ability of ENU-induced mutations to become fixed. O'Connor (24) has shown that mitotic activity in small intestinal crypts of White Swiss mice from days 13 to 19 after birth is 25% lower than between days 5 and 12. This decrease in mitotic activity could be one explanation for the decline in tumor multiplicity seen in *Min*/+ mice treated at 15 to 19 days of age relative to mice treated at 5 to 14 days of age. However, mitotic activity at all times during the first 3 weeks of life is lower than that from day 21 to adulthood (20, 24). There is also a gradual decline in the apparent cell cycle time in the small intestine from birth to adulthood (20, 24). These observations suggest that changes in the intestinal mitotic index and/or cell cycle time are not likely explanations for elevated susceptibility of young *Min*/+ mice to tumor induction by ENU. However, it must be noted that these measurements of mitotic index and cell cycle time in intestinal crypts include both the stem cells and their transitory daughter cells. Direct measurement of these kinetic parameters in the stem cells alone during intestinal development will be required to determine if these factors might influence the timing of intestinal tumor initiation.

Another possible explanation for differences in susceptibility to ENU-induced tumors is an age-related difference in DNA repair systems. Nakatsuru *et al.* (25) have shown that O^6-methylguanine DNA methyltransferase activity in the liver of 3-week-old B6 mice is lower than in 30-week-old mice. Whether there is a correlation between age and ability to repair DNA damage in the intestinal stem cell is not known.

Other factors, including hormonal influences, changes in intestinal microflora, and immunological status, may also be important in the enhanced susceptibility of young mice to intestinal tumor induction. It has been postulated that the immune system might play a role in inhibiting tumor formation by eliminating cells that express tumor-specific antigens (26). Interestingly, very low levels of immunocytes have been reported in the intestine of C3H mice during the first 2 weeks of life (27). The immunosurveillance capacity of the intestine might, therefore, be partially compromised during this developmental period in the mouse.

Whichever biological factor(s) contribute to the age-related susceptibility to intestinal tumor initiation, it seems clear that the proportion of tumors arising in each region of the intestinal tract is independent of ENU treatment. The increased total tumor number seen in ENU-treated mice was distributed along the length of the intestinal tract in the same proportions as observed in untreated *Min*/+ mice (Fig. 2). Except for the difference noted for mice treated at 5 to 9 *versus* 20 to 25 days of age, the distribution of tumor number throughout the intestine in ENU-treated mice was also not affected by the age at treatment (Fig. 2).

ENU is believed to exert its carcinogenic effect by causing point mutations and, to a lesser degree, small deletions (28–29). There are numerous gene targets upon which ENU could be acting to cause an increase in the number of intestinal adenomas in *Min*/+ mice. One of the earliest somatic genetic events involved in intestinal tumor formation for both humans with familial adenomatous polyposis and *Min*/+ mice is mutation or loss of the wild-type *APC/Apc* allele (30–32). In humans, both germline and somatic *APC* mutations tend to be clustered in the first one-half of the gene, and almost invariably cause truncation of the gene product (33). Intestinal tumors from untreated *Min*/+ mice all show loss of the chromosome 18 homologue, which carries the wild-type *Apc* allele (30, 31). In addition to *Apc*, murine chromosome 18 carries *Mcc* (mutated in colorectal cancer) and *Dcc* (deleted in colorectal carcinomas), two other genes for

which loss of function has been implicated in human intestinal cancer (32, 34). Other potential targets for ENU activity include the K-*ras* proto-oncogene and *p53* tumor suppressor gene, which have been shown to be mutated in human intestinal tumors (32), and the *Mom-1* (modifier of min) locus, which is known to strongly influence tumor multiplicity in *Min*/+ mice (11). Whether ENU treatment results in intragenic mutation of the wild-type *Apc* allele and/or causes mutations in other loci known to be involved in intestinal tumor formation is currently under investigation.

The relationship between age at ENU treatment and cystic crypt multiplicity was clearly distinct from that seen for intestinal tumors (Fig. 1*B*). The smallest increase in the number of cystic crypts observed 65 days after ENU treatment occurred in mice treated at 5 to 9 days of age. The average number of cysts was significantly higher for mice treated at all other ages. The relatively small increase in the number of cystic crypts and large increase in the number of tumors for mice treated at 5–9 days of age is consistent with a hypothesis of cystic crypts being converted into neoplastic lesions. However, mice treated at 10–14 days of age had high numbers of both cystic crypts and tumors (Fig. 1). In addition, mice treated at 30–35 days of age continued to develop a high number of cystic crypts, yet tumor multiplicity had declined significantly relative to mice treated at younger ages (Fig. 1). These results argue against a neoplastic fate for intestinal cystic crypts. The distribution of cystic crypts along the length of the intestinal tract was also shown to be significantly different from that seen for intestinal adenomas (Fig. 2).

Oshima *et al.* (35) have recently reported intestinal lesions, which they term microadenomas, in mice heterozygous for a targeted truncation mutation in the codon 716 of the *Apc* gene ($Apc^{\Delta 716}$). These microadenomas are strikingly similar to cystic crypts in histological appearance; they were also reported to arise predominantly in the small intestine of the $Apc^{\Delta 716/+}$ mice. It is noteworthy that this group also reports that microadenomas show loss of the wild-type *Apc* allele.

Recent studies of small, benign human colorectal lesions revealed two distinct molecular classes. *APC* and K-*RAS* mutations were associated with dysplastic lesions, whereas mutation of K-*RAS* alone was common in nondysplastic lesions, which seemed to have limited neoplastic potential (36). It will be important to learn if cystic crypts in *Min*/+ mice have aquired *Apc* and/or K-*ras* mutations and what the potential fate of these lesions might be.

Min/+ mice treated before 25 days of age also had a high incidence of epidermoid cysts. Terracini and Testa (4) described similar lesions in (C57BL × C3Hf)F_1 mice that were treated with methylnitrosourea at birth, but these lesions were not seen in mice treated at 35 days of age. The high incidence of these lesions in *Min*/+ mice treated with ENU before 25 days of age is particularly noteworthy since epidermoid cysts are a common extracolonic manifestation in humans with familial adenomatous polyposis (37). Although not considered a health risk, these lesions are often important in the diagnosis of this disease (37). The induction of epidermoid cysts by treatment of *Min*/+ mice with ENU suggests that somatic events are required for the development of these lesions. It will be interesting to learn if these somatic event(s) include loss or mutation of the wild-type allele of *Apc*.

In summary, we have demonstrated that early postnatal life is a time of enhanced susceptibility to intestinal adenoma induction by ENU in both *Min*/+ and +/+ mice. ENU treatment of *Min*/+ mice also increased the numbers of cystic intestinal crypts, but susceptibility to these lesions remained high up to 35 days of age and their distribution along the length of the intestinal tract was quite different from that seen for intestinal tumors. A high percentage of *Min*/+ mice treated with ENU before 25 days of age also developed epidermoid cysts.

ACKNOWLEDGMENTS

Ellen Mattes performed preliminary experiments that provided the impetus to pursue the work described here. Expert assistance with histological preparations was provided by Harlene Edwards, Dana Olson, and Jane Weeks. Dr. Henry Pitot provided assessment of histological samples as well as helpful comments on the manuscript. Melanie McNeley and Natalie Borenstein provided assistance in maintaining and genotyping animals, respectively. We also thank Dr. Norman Drinkwater, Karen Gould, and Cindy Luongo for helpful critique and Linda Clipson and Terrill Stewart for assistance in preparation of the figures.

REFERENCES

1. Vesselinovitch, S. D., Rao, K. V. N., Mihailovich, N., Rice, J. M., and Lombard, L. S. Development of broad spectrum of tumors by ethylnitrosourea in mice and the modifying role of age, sex, and strain. Cancer Res., *34:* 2530- 2538, 1974.
2. Diwan, B. A., and Blackman, K. E. Differential susceptibility of 3 sublines of C57BL/6 mice to the induction of colorectal tumors by 1,2- dimethylhydrazine. Cancer Lett., *9:* 111–115, 1980.
3. Diwan, B. A., and Meir, H. Strain- and age-dependent transplacental carcinogenesis by 1-ethyl-1-nitrosourea in inbred strains of mice. Cancer Res., *34:* 764–770, 1974.
4. Terracini, B., and Testa, M. Carcinogenicity of a single administration of *N*-nitrosomethylurea: a comparison between newborn and 5-week-old mice and rats. Br. J. Cancer, *24:* 588–598, 1970.
5. Oomen, L. C. J. M., van der Valk, M. A., Hart, A. A. M., Demant, P., and Emmelot, P. Influence of mouse major histocompatibility complex (H-2) on *N*-ethyl-*N*-nitrosourea-induced tumor formation in various organs. Cancer Res., *48:* 6634–6641, 1988.
6. Searle, C. E., and Jones, E. L. The multipotential carcinogenic action of *N*-ethyl-*N*-nitrosourea administered neonatally to mice. Br. J. Cancer, *33:* 612–625, 1976.
7. Searle, C. E., and Jones, E. L. Tumors of the nervous system in mice treated neonatally with *N*-ethyl-*N*-nitrosourea. Nature (Lond.), *240:* 559–560, 1972.
8. Su, L-K., Kinzler, K. W., Vogelstein, B., Preisinger, A. C., Moser, A. R., Luongo, C., Gould, K. A., and Dove, W. F. A germline mutation of the murine homolog of the *APC* gene causes multiple intestinal neoplasia. Science (Washington DC), *256:* 668–670, 1992.
9. Moser, A. R., Pitot, H. C., and Dove, W. F. A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science (Washington DC), *247:* 322–324, 1990.
10. Moser, A. R., Dove, W. F., Roth, K. A., and Gordon, J. I. The *Min* (Multiple Intestinal Neoplasia) mutation: its effect on gut epithelial cell differentiation and interaction with a modifier system. J. Cell Biol., *116:* 1517–1526, 1992.
11. Dietrich, W. F., Lander, E. S., Smith, J. S., Moser, A. R., Gould, K. A., Luongo, C., Borenstein, N., and Dove, W. F. Genetic identification of *Mom- 1*, a major modifier locus affecting *Min*-induced intestinal neoplasia in the mouse. Cell, *75:* 631–639, 1993.
12. Moser, A. R., Mattes, E. M., Dove, W. F., Lindstrom, M. J., Haag, J. D., and Gould, M. N. Apc^{Min}, a mutation in the murine *Apc* gene, predisposes to mammary carcinomas and focal alveolar hyperplasias. Proc. Natl. Acad. Sci. USA, *90:* 8977–8981, 1993.
13. Hollander, M., and Wolfe, D. A. Nonparametric Statistical Methods. New York: John Wiley and Sons, Inc., 1973.
14. Walpole, R. E., and Myers, R. H. Probability and Statistics for Engineers and Scientists, Ed. 3. New York: Macmillan, Inc., 1985.
15. Ehrenberg, L., and Wachtmeister, C. A. Handling of mutagenic chemicals: experimental safety. *In:* B. J. Kilby, M. Lagator, W. Nichols, and C. Ramel (eds.), Handbook of Mutagenicity Test Procedures, Ed. 2, pp. 765–774. Amsterdam: Elsevier, 1984.
16. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S., Jung, W. E., Li, E., Weinberg, R. A., and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. Cell, *81:* 197–205, 1995.
17. Potten, C. S., and Loeffler, M. Stem cells: attributes, cycles, spirals, pitfalls and uncertainties—lessons for and from the crypt. Development (Camb.), *110:* 1001–1020, 1990.
18. Ponder, B. A. J., Schmidt, G. H., Wilkinson, M. M., Wood, M. J., Monk, M., and Reid, A. Derivation of mouse intestinal crypts from single progenitor cells. Nature (Lond.), *313:* 689–691, 1985.
19. Schmidt, G. H., Winton, D. J., and Ponder, B. A. J. Development of the pattern of cell renewal in the crypt-villus unit of chimeric mouse small intestine. Development (Camb.), *103:* 785–790, 1988.
20. Al-Nafussi, A. I., and Wright, N. A. Cell kinetics in the mouse small intestine during immediate postnatal life. Virchows Arch. Cell Pathol., *40:* 51–62, 1982.
21. Maskens, A. P., and Dujardin-Loits, R-M. Kinetics of tissue proliferation in colorectal mucosa during post-natal growth. Cell Tissue Kinet., *14:* 467–477, 1981.
22. St. Clair, W. H., and Osborne, J. W. Crypt fission and crypt number in the small and large bowel of postnatal rats. Cell Tissue Kinet., *18:* 255–262, 1985.
23. Cairnie, A. B., and Millen, B. H. Fission of crypts in the small intestine of the irradiated mouse. Cell Tissue Kinet., *8:* 1189–1196, 1975.
24. O'Connor, T. M. Cell dynamics in the intestine of the mouse from late fetal life to maturity. Am. J. Anat., *118:* 525–536, 1966.
25. Nakatsuru, Y. Aoki, K., and Ishikawa, T. Age and strain dependence of O^6-methylguanine DNA methyltransferase activity in mice. Mutation Res., *219:* 51–56, 1989.

26. Doherty, P. C., Knowles, B. B., and Wettstein, P. J. Immunological surveillance of tumors in the context of major histocompatibility complex restriction of T cell function. Adv. Cancer Res., *42:* 1–65, 1984.
27. Crabbé, P. A., Nash, D. R., Bazin, H., Eyssen, H., and Heremans, J. F. Immunohistochemical observations on lymphoid tissue from conventional and germ-free mice. Lab. Invest., *22:* 448–457, 1970.
28. Eckert, K. A., Ingle, C. A., Klinedinst, D. K., and Drinkwater, N. R. Molecular analysis of mutations induced in human cells by *N*-ethyl-*N*-nitrosourea. Mol. Carcinog., *1:* 50–56, 1988.
29. Skopek, T. R., Walker, V. E., Cochrane, J. E., Craft, T. R., and Cariello, N. F. Mutational spectrum at the *Hprt* locus in splenic T cells of B6C3F1 mice exposed to *N*-ethyl-*N*-nitrosourea. Proc. Natl. Acad. Sci. USA, *89:* 7866–7870, 1992.
30. Luongo, C., Moser, A. R., Gledhill, S., and Dove, W. F. Loss of Apc^+ in intestinal adenomas from Min mice. Cancer Res., *54:* 5947–5952, 1994.
31. Levy, D. B., Smith, K. J., Beazer-Barclay, Y., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Inactivation of both *APC* alleles in human and mouse tumors. Cancer Res., *54:* 5953–5958, 1994.
32. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell, *61:* 759–767, 1990.
33. Nagase, H., and Nakamura, Y. Mutations of the *APC* (adenomatous polyposis coli) gene. Human Mutat., *2:* 425–434, 1993.
34. Luongo, C., Gould, K. A., Su, L-K., Kinzler, K. W., Vogelstein, B., Dietrich, W., Lander, E. S., and Moser, A. R. Mapping of multiple intestinal neoplasia (Min) to proximal chromosome 18 of the mouse. Genomics, *15:* 3–8, 1993.
35. Oshima, M., Oshima, H., Kitagawa, K., Kobayashi, M., Itakura, C., and Taketo, M. Loss of *Apc* heterozygosity and abnormal tissue building in nascent intestinal polyps in mice carrying a truncated *Apc* gene. Proc. Natl. Acad. Sci. USA, *92:* 4482–4486, 1995.
36. Jen, J., Powell, S. M., Papadopoulus, N., Smith, K. J., Hamilton, S. R., Vogelstein, B., and Kinzler, K. W. Molecular determinants of dysplasia in colorectal lesions. Cancer Res., *54:* 5523–5526, 1994.
37. Jagelman, D. G. Extracolonic manifestations of familial polyposis coli. Semin. Surg. Oncol., *3:* 88–91, 1987.