normal liver-derived GP96 and monitored for long periods for gross measures such as weight loss, ruffling of fur, life-span, and more specific parameters such as hepatotoxicity, no adverse consequences indicative of autoimmune phenomena have been detected (*20*). Further, in a recent phase I study, in which patients with progressive malignancies were immunized with autologous cancer-derived GP96 preparations, patients were followed for over a year specifically for signs of autoimmunity. No such signs were detected (*19*).

Regardless of the method of their induction or their lack of intentional induction, cancers are antigenically individually distinct (*21*, *22*). This antigenic repertoire may consist of peptides that are rendered antigenic by the mutations within cancers; because the mutations are random and many, a repertoire becomes a fingerprint, which has little probability of repeating itself (*1*, *22*). Immunization with cancer-derived HSP preparations permits access to the antigenic fingerprint of a cancer without a need for identification of this repertoire for each patient's cancer. Although immunization with defined cytolytic T lymphocyte epitopes that are shared between different cancers is widely perceived as a potential method of generating common cancer vaccines (*23*, *24*), immunization with such epitopes may not elicit protective tumor immunity (*25*). Thus, therapies based on the complex, dynamic, and individually unique repertoire of tumor antigens may have advantages for treatment of cancer (*1*, *22*).

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26. All studies involving mice were carried out under protocols approved by the Institutional Animal Care and Use Committee of Fordham University.
27. Antibodies against GP96 (9G10.F8.2) and HSP70 (BRM22) were obtained from NeoMarkers (Fremont, CA).
28. Mice were depleted of $CD4^+$ or $CD8^+$ T cells by intraperitoneal (ip) injection of 250 μl of 1:8 diluted GK1.5 ascites or YTS-169.4 ascites, respectively. Injections were continued biweekly. NK cells were depleted by ip injection of 200 μg of purified monoclonal antibody NK1.1 every week. Mice in the control group were injected with 200 μg of rat or mouse Ig once per week. The efficiency of depletion was confirmed by flow cytometry, and the specific depletion was 90 to 100%.

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Rapid Colorectal Adenoma Formation Initiated by Conditional Targeting of the *Apc* Gene

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Familial adenomatous polyposis coli (FAP) is a disease characterized by the development of multiple colorectal adenomas, and affected individuals carry germline mutations in the *APC* gene. With the use of a conditional gene targeting system, a mouse model of FAP was created that circumvents the embryonic lethality of Apc deficiency and directs *Apc* inactivation specifically to the colorectal epithelium. *loxP* sites were inserted into the introns around *Apc* exon 14, and the resultant mutant allele (Apc^{580S}) was introduced into the mouse germline. Mice homozygous for Apc^{580S} were normal; however, upon infection of the colorectal region with an adenovirus encoding the Cre recombinase, the mice developed adenomas within 4 weeks. The adenomas showed deletion of *Apc* exon 14, indicating that the loss of Apc function was caused by Cre-*loxP*–mediated recombination.

Mutations in the *APC* gene are responsible for FAP (*1*), a disease characterized by the development of thousands of colorectal adenomas (*1*). FAP patients have germline *APC* mutations, and their tumors show inactivation of the wild-type allele (*2*). Inactivation of both *APC* alleles also occurs frequently in sporadic colorectal adenomas (*3*). These results suggest that *APC* is a tumor suppressor gene, although the mechanism by which *APC* mutation leads to transformation of colorectal epithelial cells is largely unknown.

Several mouse lines carrying *Apc* mutations in their germline have been established as experimental models for FAP (*4*). For example, the Min mouse, which has a germline mutation in *Apc*, develops more than 100 intestinal adenomas, a subset of which show inactivation of the second *Apc* allele (*4*, *5*). However, most of the tumors in the Min mouse develop in the small intestine rather than the colon (*5*), and it is difficult to study the early events in tumorigenesis in this model.

To study the initiation stage of colon

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adenoma formation, we have developed an *Apc* mutant mouse in which inactivation of *Apc* function is achieved by conditional targeting. This model is based on the Cre-*loxP* recombination system (6). We first introduced a pair of *loxP* sites into introns 13 and 14 of the *Apc* gene by targeted mutagenesis (Fig. 1A) (7). The targeting vector was introduced into embryonic stem (ES) cells by electroporation, and two homologous recombinant ES clones, cl 66 and 76, were obtained (Fig. 1B) (8). The results of Southern (DNA) blot analyses confirmed that these clones carry a mutant *Apc* allele that is produced by homologous recombination with the targeting vector (Fig. 1C) (9). Two independent mutant mouse lines were established from the clones by blastocyst injection and subsequent breeding of chimeras (7).

Recombination mediated by the Cre recombinase deletes a region of *Apc* encompassing exon 14 and induces a frameshift mutation at codon 580. We therefore named this silent mutant allele Apc^{580S} (Fig. 1A). No tumors were observed in Apc^{580S} heterozygotes after 1 year. Moreover, although homozygous Apc^{Min} mutation typically causes embryonic lethality (10), 66 of the 253 F_2 offspring obtained by double heterozygous breeding of F_1 mice were identified as homozygous mutants of Apc^{580S} without any apparent phenotype (Fig. 1D) (11). This percentage of homozygotes (26.1%) is close to that expected from Mendelian inheritance. Northern (RNA) blot analysis revealed that *Apc* expression in the intestinal tract of homozygous mutants was ~30% of that in wild-type mice (Fig. 1E) (12). This was the only phenotypic alteration observed in Apc^{580S} mice, and an equivalent reduction in the amount of *Apc* mRNA was not detected in the brain (Fig. 1E). We also analyzed Apc protein expression in the colon of Apc^{580S} homozygotes by immunohistochemistry and found the staining pattern to be indistinguishable from that of wild-type mice (Fig. 1F) (13). The Apc^{580S} homozygotes developed normally and had no detectable tumors over a 1-year period. We therefore concluded that the insertion of the *loxP* sites and the PGKneo cassette into the *Apc* introns did not impair *Apc* functions in the mice and that Apc^{580S}

Table 1. Colorectal tumor incidence in mice infected by rectal infusion with recombinant adenoviruses encoding the Cre recombinase. Tumor incidence is expressed as number of mice with tumors/number infected. ND, not done.

Genotype of infected mice	Virus	Tumor incidence	
		4 weeks	3 months
Apc^{580S}/Apc^{580S}	AxCANLacZ	ND	0/15
Apc^{580S}/Apc^{580S}	AxSRαCre	ND	3/5
Apc^{580S}/Apc^{580S}	AxCANCre	20/24	4/5
$Apc^{580S}/+$	AxCANCre	0/14	ND
+/+	AxCANCre	0/13	ND

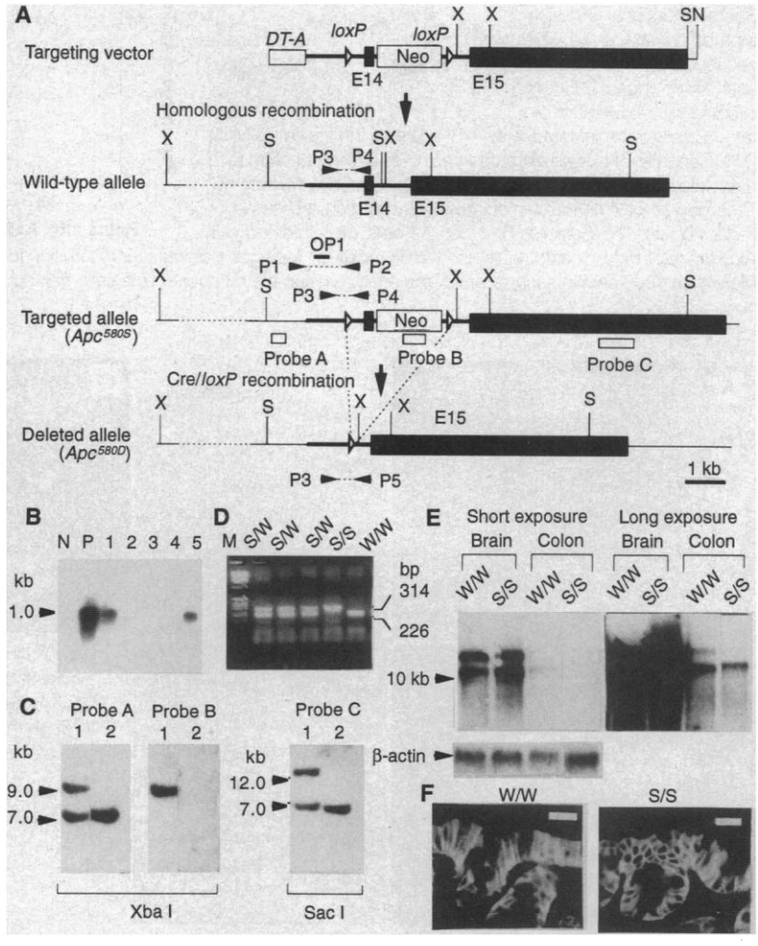

Fig. 1. Establishment of a mutant mouse line carrying a conditionally targeted *Apc* allele. (**A**) Structure of the conditionally targeted allele of *Apc* (Apc^{580S}) and the mutant *Apc* allele resulting from the deletion mediated by Cre-*loxP* recombination (deleted allele, Apc^{580D}). The targeting vector was constructed by inserting one *loxP* site into intron 13 and the other, with a PGK-neo cassette (Neo), into intron 14, resulting in exon 14 (E14) flanked by a pair of *loxP* sites (open triangles) (7). Positions of PCR primers used for the detection of each allele are indicated (P1 to P5). Positions of probes used for Southern blot analysis with Xba I (X) or Sac I (S) are also shown (probes A to C). (**B**) PCR analysis of the ES clones (8). DNA of J1 ES cells was used as a negative control (N) and DNA of plasmid pApc3.0 as a positive control (P) (8). Positive signals are evident for clones 66 and 76 (lanes 1 and 5), but not for the negative clones (lanes 2 to 4). (**C**) Southern blot analysis of ES clones (9). ES cell DNA was digested with Xba I or Sac I and hybridized with three different probes derived from the *Apc* gene (probes A and C) or a neo cassette (probe B). The results shown for cl 66 (lane 1) and J1 ES cells (lane 2) are representative. (**D**) Genotypes of F_2 offspring obtained by double heterozygous breeding of Apc^{580S} F_1 mice (11). Tail DNAs were analyzed by PCR using primers P3 and P4. The wild-type allele (W) generates a band of 226 bp, and Apc^{580S} (S) generates a band of 314 bp. The results shown for five offspring—one homozygous mutant (S/S), three heterozygotes (S/W), and one wild-type mouse (W/W)—are representative. (**E**) Analysis of *Apc* expression in Apc^{580S} mice by Northern blotting (12). RNA extracted from the brains or colons of Apc^{580S} homozygotes (S/S) or wild-type mice (W/W) were analyzed. Autoradiograms obtained with two different exposures are shown. The amount of RNA applied was quantified by probing with β-actin cDNA. (**F**) Immunohistochemical analysis of Apc protein expression in the colon of Apc^{580S} homozygotes (S/S) or wild-type mice (W/W) (13). Scale bars, 30 μm.

could serve as a conditional mutant.

We next established a system that allowed controlled expression of Cre recombinase in the intestinal epithelium of Apc^{580S} mice. Recombinant adenoviruses are efficient agents for gene delivery; they have broad tissue specificity, and the expression of the delivered gene is transient, because the virus does not integrate into the host genome (*14*). This feature is important because constitutive expression of the Cre recombinase after recombination may in itself modify the cellular phenotype.

The Cre recombinase gene, driven by the SRα promoter, was introduced into an adenovirus vector from which the E1A and E1B genes had been deleted, and the virus (called AxSRαCre) was propagated in 293 cells, which endogenously express the E1A gene product (*15*). The function of the virally expressed recombinase was initially analyzed in cultured cells. β-geo 42 cells, established from NIH 3T3 cells by gene trapping, carry a copy of a recombinant provirus containing splice acceptor sequences (SA) and a fusion gene (β-geo) consisting of the neomycin resistance gene (*neo*) and β-galactosidase gene (*lacZ*) as the gene-trap machinery, flanked by a pair of *loxP* sites located in 5′ and 3′ long terminal repeats (LTRs) (Fig. 2A) (*16*). This cell line was infected with AxSRαCre (*17*), and the results of Southern blot analysis confirmed the deletion of the provirus from the genome of the infected β-geo 42 cells (Fig. 2B). DNA fragments specific for the expected deletion (4.3 and 6.2 kb) were detected in the AxSRαCre-infected cells when the multiplicity of infection (MOI) was ≥1.25, and almost all cells infected at an MOI of 30 had the expected deletion (Fig. 2B). Examination of *lacZ* expression (*18*) in the infected cells revealed that 100% of the cells had lost *lacZ* expression within 48 hours after infection at a MOI of ≥30 (Fig. 2C). These results indicate that the virus induced Cre-*loxP*–mediated recombination immediately after infection.

Before introduction of AxSRαCre into Apc^{580S} mice, we infected cl 66 cells car-

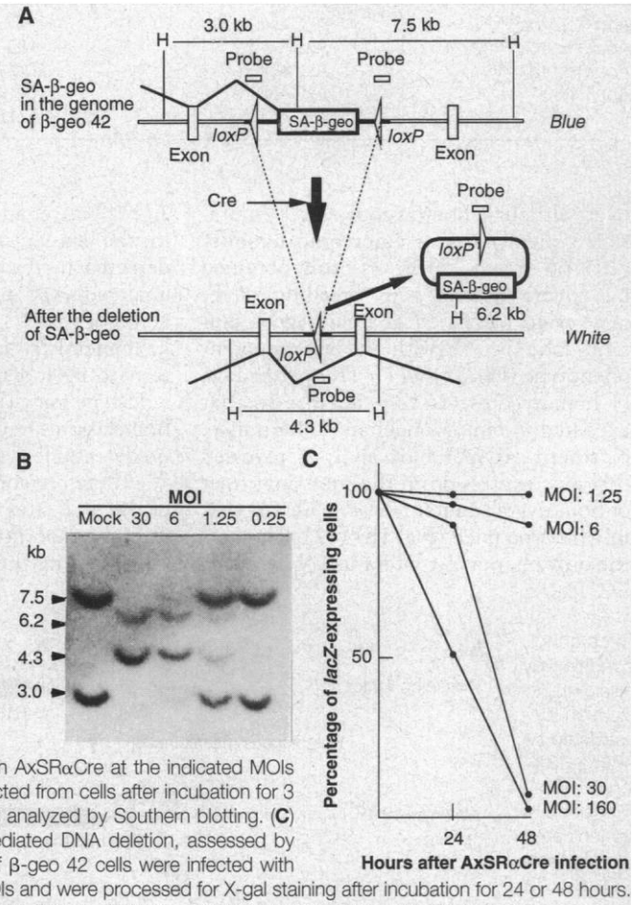

Fig. 2. Analysis of AxSRαCre, a recombinant adenovirus expressing Cre recombinase. (**A**) Structure of the gene trap machinery inserted into the genome of β-geo 42 cells. A provirus, consisting of SA and a β-geo gene flanked by a pair of *loxP* sites located in LTRs, was integrated in the cellular DNA and expressed by trapping the expression of the endogenous gene (*16*). Therefore, these cells were blue when stained with 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal). The region flanked by a pair of *loxP* sites is deleted as circular DNA from the genomic DNA by the expression of Cre recombinase. This recombination event can be detected by Southern blot analysis using a probe containing the *loxP* sequence. (**B**) Southern blot analysis of β-geo 42 cells infected with AxSRαCre. β-geo 42 cells were infected with AxSRαCre at the indicated MOIs (*17*). Genomic DNA was extracted from cells after incubation for 3 days, digested by Hind III, and analyzed by Southern blotting. (**C**) Time course of AxSRαCre-mediated DNA deletion, assessed by X-gal staining (*18*). Samples of β-geo 42 cells were infected with AxSRαCre at the indicated MOIs and were processed for X-gal staining after incubation for 24 or 48 hours. More than 1000 cells in each sample were analyzed, and the number of blue cells was counted.

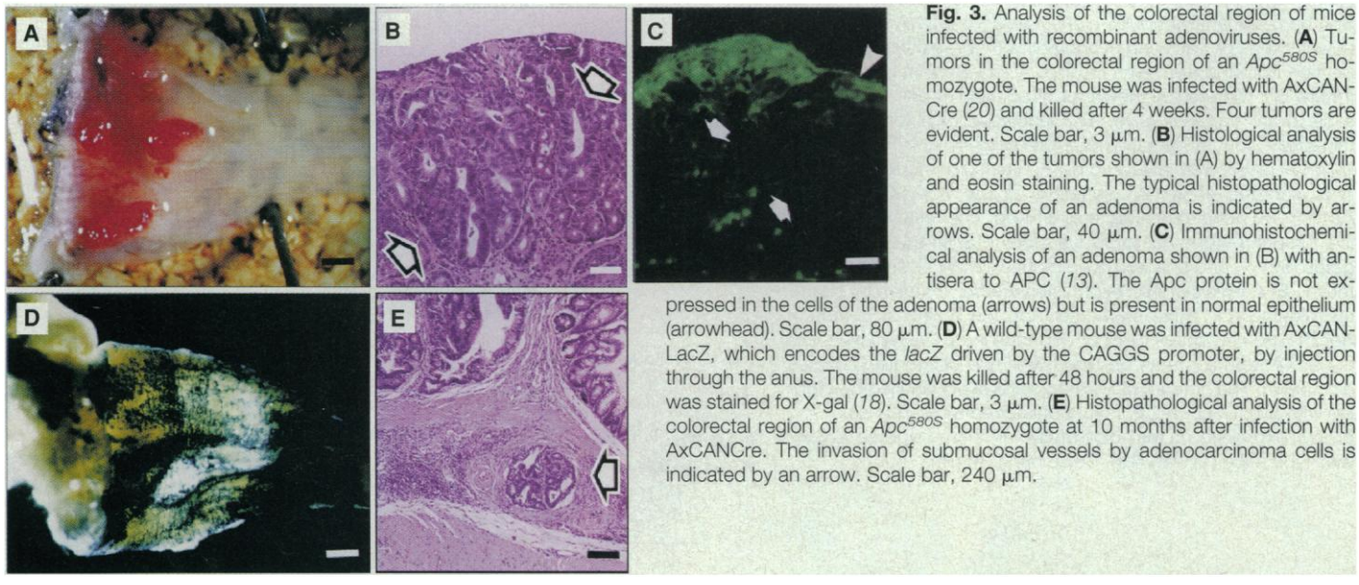

Fig. 3. Analysis of the colorectal region of mice infected with recombinant adenoviruses. (**A**) Tumors in the colorectal region of an Apc^{580S} homozygote. The mouse was infected with AxCAN-Cre (*20*) and killed after 4 weeks. Four tumors are evident. Scale bar, 3 μm. (**B**) Histological analysis of one of the tumors shown in (A) by hematoxylin and eosin staining. The typical histopathological appearance of an adenoma is indicated by arrows. Scale bar, 40 μm. (**C**) Immunohistochemical analysis of an adenoma shown in (B) with antisera to APC (*13*). The Apc protein is not expressed in the cells of the adenoma (arrows) but is present in normal epithelium (arrowhead). Scale bar, 80 μm. (**D**) A wild-type mouse was infected with AxCAN-LacZ, which encodes the *lacZ* driven by the CAGGS promoter, by injection through the anus. The mouse was killed after 48 hours and the colorectal region was stained for X-gal (*18*). Scale bar, 3 μm. (**E**) Histopathological analysis of the colorectal region of an Apc^{580S} homozygote at 10 months after infection with AxCANCre. The invasion of submucosal vessels by adenocarcinoma cells is indicated by an arrow. Scale bar, 240 μm.

rying the Apc^{580S} allele to confirm that Cre expression would induce the expected deletion (*17*). Of 20 subclones isolated after infection, 10 had the expected deletion of *Apc* exon 14, as assessed by Southern blotting and sequence analysis of genomic polymerase chain reaction (PCR) products (*19*). We next generated a mutant mouse line by injecting one of these clones into blastocysts. Reverse transcriptase–mediated PCR analysis of RNA extracted from intestine and brain of the mutant mouse carrying this deleted allele, Apc^{580D}, confirmed the deletion of the region encoded by exon 14 from the Apc^{580D} transcripts, and resulted in a frame-shift mutation (*19*). All Apc^{580D} heterozygotes developed multiple intestinal tumors, and Apc^{580D} homozygotes died during embryogenesis (*19*). These results indicate that *Apc* function is greatly impaired by the Cre-*loxP*–mediated deletion.

We next attempted to induce *Apc* mutation by expressing Cre recombinase in colorectal epithelial cells of Apc^{580S} homozygotes. For these experiments, we used both AxCANCre (*20*), an adenovirus that encodes the Cre enzyme with an artificial nuclear localization signal, and AxSRαCre. The viruses were injected into the colorectum of Apc^{580S} homozygotes through the anus (*21*), and tumor formation was analyzed by necropsy after 3 months. Homozygous mutants infected with AxCANLacZ (*20*), a recombinant adenovirus expressing the *lacZ* gene, did not develop any tumors. Colorectal adenomas developed in 80% of the Apc^{580S} homozygotes infected with AxCANCre (7.1 tumors per mouse on average) and in 60% of those infected with AxSRαCre (2.2 tumors per mouse on average) (Table 1).

To analyze earlier stages of adenoma formation, we next administered AxCANCre to Apc^{580S} homozygotes, heterozygotes, and wild-type mice, and analyzed them 4 weeks after infection. Colorectal tumors were observed in 20 of 24 Apc^{580S} homozygotes (Table 1) (Fig. 3A), but were not seen in any of the 14 heterozygotes nor in any of the 13 wild-type mice. All of the tumors were diagnosed as adenomas by histopathology (Fig. 3B), and immunohistochemical analysis (*13*) showed that intact Apc protein was not expressed in the adenoma cells (Fig. 3C). Scrape-PCR analysis of genomic DNA from these adenomas confirmed that all adenomas had lost the Apc^{580S} allele and contained the Apc^{580D} allele (Fig. 4). This finding suggested that Cre-*loxP*–mediated inactivation of both *Apc* alleles was responsible for adenoma development.

The average number of adenomas of the Apc^{580S} homozygotes treated with AxCANCre was 6.7, all occurring within ~3 cm of the anal ring. This localization may be a result of our infection procedure. When we infected the colorectal region of wild-type mice (*21*) with AxCANLacZ, we found that essentially the same region was stained blue (Fig. 3D). About 10 to 20% of the cells displayed enzyme activity, indicating efficient expression of the gene transferred by AxCANLacZ. On this basis, we infer that *Apc* inactivation by AxCANCre infection may have occurred in a similar percentage of cells. The fact that relatively few adenomas were generated suggests that another event may be necessary for adenoma formation.

When we followed up homozygous mutants that were allowed to live after infection, five of six mice survived more than 1 year, possibly because of the regional localization of adenoma formation. By pathological analysis at necropsy, about 50% of their tumors showed invasion of the submucosal layer by tumor cells (Fig. 3E) and were diagnosed as adenocarcinomas. The conditional gene targeting system described here may allow chronological analysis of adenoma formation in a more precise manner than that allowed by other mouse models.

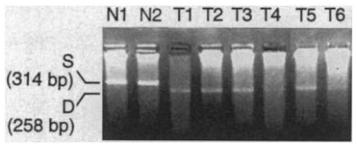

Fig. 4. Molecular analysis of the mutant *Apc* allele of adenomas. Genomic DNA was extracted from six adenomas (T1 to T6) and adjacent normal tissues (N1 and N2) by scraping paraffin sections of the colorectal region of two different Apc^{580S} homozygotes infected with AxCANCre; analysis was by PCR using primers P3, P4, and P5 (*23*). In all adenomas, DNA fragments of 258 bp, specific for Apc^{580D} (D), are evident with complete disappearance of those of 314 bp, specific for Apc^{580S} (S).

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7. The targeting vector was constructed as follows: A 129/Sv mouse genomic DNA fragment of 10.0 kb, containing *Apc* exons 14 and 15 and introns 13 and 14, was isolated. A synthetic oligonucleotide, LoxP-Brn5, containing a *loxP* sequence (5′-ATAACTTCGTATAGCATACATTATACGAAGTTAT-3′) attached to a unique sequence (5′-ACCAAACCCGGGCTTTGCTGAC-3′), was inserted into the Hinc II site in intron 13. The PGKneo cassette containing its own polyadenylation sequence, attached to another oligonucleotide with a *loxP* sequence at the 3′ end, was inserted into the Sac I site in intron 14. A pMC1-DT-A cassette was also attached. Before electroporation, the targeting vector was linearized at the Not I site. Electroporation and G418 selection of J1 ES cells were carried out as in (*22*). Homologous recombinant ES cells were identified and injected into blastocysts (*22*). F_1 mice were obtained by breeding chimeric males with C57/BL6J females. All animal work was carried out in accordance with institutional guidelines.
8. Genomic DNAs of 471 G418-resistant ES clones were screened by PCR using a primer pair, P1 (5′-GCTCTGTGTGTCTGCATAAGACCCTGG-3′) and P2 (5′-GTCAGCAAAGCCCGGGTTTGGTGGT-3′). PCR products were analyzed by Southern blotting using ^{32}P end-labeled synthetic oligonucleotide OP1 (5′-CTCTCTTACATTATCTGCGTTATCC-3′) as a probe. Plasmid pApc3.0, which carries a LoxP-Brn5 oligonucleotide inserted into the Hinc II site of the 3.0-kb Sac I fragment, was used as a template for positive control.
9. Southern blot analysis (*22*) was performed with the following probes: probe A, a 0.4-kb Nco I fragment derived from *Apc* intron 13; probe B, a 0.6-kb Pst I fragment of *neo*; and probe C, a 1.0-kb Hind III–Xba I fragment derived from *Apc* exon 15.
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11. Genotyping of 4-week-old F_2 offspring was carried out by PCR analysis of tail DNA using P3 (5′-GTTCTGTATCATGGAAAGATAGGTGGTC-3′) and P4 (5′-CACTCAAAACGCTTTTGAGGGTTGATTC-3′) primers.
12. Polyadenylated RNAs (5 μg) extracted from the brains or colons of mice were analyzed by Northern blotting. A fragment of mouse *Apc* cDNA (nucleotides 33 to 920) or mouse β-actin cDNA was used as the probe.
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17. β-geo 42 cells were inoculated with virus solution for 1 hour. Different MOIs were achieved by serial dilution of the stock solution (5×10^8 pfu/ml). The cl 66 ES cells were suspended at 2×10^5 cells/ml, and the stock solution of AxSRαCre was added at a MOI of 1.5. After inoculation for 30 min, infected cells were cultured for 6 days and subcloned.
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21. Eight- to 12-week-old mice, kept under starvation for 2 days, were anesthetized. The colorectal region was cleaned by cotton swabs inserted through the anus after three successive enemas of saline (1 ml). Adenovirus solution (0.3 ml, 5×10^8 pfu/ml) was infused through the anus.
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23. The P5 primer is 5′-GAGTACGGGGTCTCTGTCTCAGTGAA-3′.

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