ent structural classes of triple helices for oligonucleotide-directed recognition of double-helical DNA, both are currently limited to binding purine-rich tracts of DNA. Moreover, the sequence-composition dependence of the triplet stabilities within each triple-helix motif remains to be elucidated. High-resolution x-ray crystallographic data for either structural class does not yet exist.

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An in Vivo Model of Somatic Cell Gene Therapy for Human Severe Combined Immunodeficiency

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Deficiency of adenosine deaminase (ADA) results in severe combined immunodeficiency (SCID), a candidate genetic disorder for somatic cell gene therapy. Peripheral blood lymphocytes from patients affected by ADA$^-$ SCID were transduced with a retroviral vector for human ADA and injected into immunodeficient mice. Long-term survival of vector-transduced human cells was demonstrated in recipient animals. Expression of vector-derived ADA restored immune functions, as indicated by the presence in reconstituted animals of human immunoglobulin and antigen-specific T cells. Retroviral vector gene transfer, therefore, is necessary and sufficient for development of specific immune functions in vivo and has therapeutic potential to correct this lethal immunodeficiency.

GENE THERAPY ASSUMES THAT A DEfinitive cure for a genetic disease should be possible by directing treatment to the abnormal gene rather than to secondary effects of its products (*1*). The altered gene can be complemented by introducing the normal gene into the cell genome with an appropriate vector. Retroviral vectors can be used to introduce exogenous DNA sequences into hematopoietic progenitors and pluripotent stem cells. Gene transfer has been successful in vitro and in vivo in the mouse (*2, 3*) and with lower efficiency in cultured hematopoietic progenitors that were derived from dogs and primates (*4, 5*), including humans (*6, 7*).

Deficiency of the enzyme adenosine deaminase (ADA) results in a variant of severe combined immunodeficiency (SCID), a lethal disorder usually treated with allogeneic bone marrow transplantation (*8*). Retroviral vectors derived from the N2 prototype can efficiently transduce the human ADA gene into established lymphoid cell lines that had been derived from ADA$^-$ SCID patients (*9*) and into hematopoietic progenitors in both short- and long-term culture, with expression of the newly introduced ADA enzyme occurring in cells of myeloid and lymphoid lineages (*7*). However, in neither system was direct evidence available that expression of ADA activity in ADA$^-$ cells would restore specific immune functions. To address this issue, we reconstituted a number of immunodeficient BNX (homozygous *bg/nu/x^{id}*) mice (*10*) with human ADA$^-$ peripheral blood lymphocytes (PBLs) transduced with a retroviral vector (DCA, double-copy ADA) that contained a human ADA mini-gene (*11*). We then analyzed human cell survival, ADA gene transfer and expression, and reconstitution of immune functions.

BNX mice were injected with DCA-in-

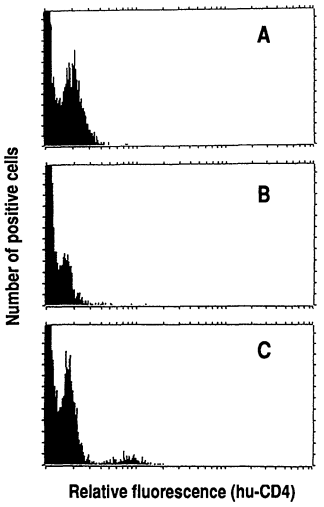

Fig. 1. Frequency of CD4$^+$ human T cells in the spleen of recipient BNX mice 6 weeks after reconstitution. (**A**) Untreated animal. (**B**) Animal treated with mock-infected ADA$^-$ PBLs. (**C**) Animal reconstituted with DCA-infected ADA$^-$ PBLs (751). Animals 721, 751, 727, and 651 had frequencies of CD4$^+$ human T cells varying between 2.1 and 4.2. All other animals had lower values. Fluorescein isothiocyanate (FITC)–conjugated MAbs to human (hu)-CD4 (T4), hu-CD8 (T8), hu-CD19 (B4), and hu-CD20 (B1) were obtained from Coulter Immunology (Hialeah, Florida). Isotype-matched MAbs were used for all analyses. Conjugated MAbs were chosen in order to reduce nonspecific binding. Furthermore, positive data were accepted only when background from negative controls (unstained cells from the experimental animal and stained cells from a mock-reconstituted animal) did not exceed 1% of positive cells. Flow analysis was done on a EPICS 751 cell sorter (Coulter Electronics, Hialeah, Florida), equipped with a 5 W Argon laser (Innova 90, Coherent Inc., Palo Alto, California).

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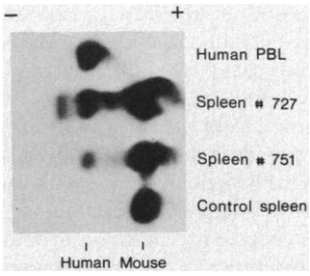

Fig. 2. Cellulose acetate electrophoresis analysis of human ADA enzyme production in spleen cells from BNX mice reconstituted with DCA-infected ADA$^-$ PBLs. Lysates were prepared from 1 × 10^7 cells in 10 μl of lysing buffer. Two microliters of cell lysate were separated on Cellogel matrix (Chemetron, Milano, Italy) and stained in situ for ADA activity (*15*). Mobilities of human and mouse enzymes are indicated. The lysate concentration was ten times higher than the standard used in this assay (*15*). At this concentration, the enzymatic assay is no longer proportional, leading to overestimation of the relative human ADA band.

fected ADA$^-$ PBLs (n = 8), mock-infected ADA$^-$ PBLs (n = 4), or normal adult PBLs (n = 20) as controls for the efficiency of reconstitution (*12*). Survival and function of human cells were monitored biweekly by assaying human immunoglobulin (hu-IgG) in recipients peripheral blood (*13*). Only one of the eight animals treated with DCA-infected ADA$^-$ PBLs had no detectable hu-IgG. All others had serum concentrations ranging from 3.7 to 672.1 mg/dl (median of 48.0; control <0.5 mg/dl). No hu-IgG was detected in the peripheral blood of recipients treated with mock-infected ADA$^-$ PBLs at 6 to 10 weeks from injection. Low concentrations of hu-IgG were observed in only one of these animals, 2 weeks after injection. All but 4 of the 20 animals reconstituted with normal PBLs had detectable hu-IgG at 6 weeks, with values ranging from 3.1 to 429.0 mg/dl (median of 29.8). Recipient animals were sacrificed 6 to 10 weeks after treatment.

Human cells were present in significant numbers (1 to 10%) in the spleen of four out of eight animals reconstituted with vector-transduced ADA$^-$ PBLs at 6 to 10 weeks, as estimated by the positive signal for human Alu sequences in spleen DNA (*14*). A similar reconstitution efficiency was observed in control mice treated with normal PBLs. Human cells did not persist in animals injected with mock-infected ADA$^-$ PBLs, suggesting that intracellular expression of ADA was necessary for survival. Few animals with detectable serum hu-IgG had no signal for Alu sequences in spleen DNA, suggesting that B and possibly T cells could either circulate or colonize different organs. Human cells were also detected by flow cytofluorimetry with monoclonal antibodies (MAbs) to human cell surface markers. Human CD4$^+$ T cells were 4.2% of the total splenocytes from a representative recipient BNX mouse 6 weeks after reconstitution (Fig. 1). The only other marker repeatedly detected at low frequency (~2%) was the human B cell marker CD20. The use of a MAb to the human leukocyte common antigen CD45 confirmed that these two cell types represented the majority of human cells in the spleen of recipient mice.

Production of human ADA in Alu$^+$ spleens from mice treated with DCA-infected cells was assayed by cellulose-acetate electrophoresis and enzymatic staining (Fig. 2). The human enzyme could be distinguished from the murine one by its different mobility (*15*) and was detected in three of the four Alu$^+$ spleens (714, 727, and 751). No human ADA band was observed in spleen lysates from animals treated with mock-infected ADA$^-$ PBLs or from control untreated mice.

We attempted to expand human T lymphocytes from spleens and peritoneal lavages of five animals reconstituted with DCA-infected ADA$^-$ PBLs, and three animals treated with mock-infected ADA$^-$ PBLs (*16*). Human T cells were obtained from the former animals but never from the latter. Expanded T cell clones were human CD3$^+$ and CD4$^+$, confirming their human nature, and HLA typing confirmed the patient's origin. Frequency of human clonable T cells varied in different reconstituted animals: spleens from two animals gave no clonable cells, spleen 651 had a low cloning efficiency (five positive out of 1200 plated wells, at 1000 splenocytes per well), whereas spleens 751 and 727 had higher cloning efficiencies. All but one (clone 92) out of 58 clones obtained from spleen 751 had functional DCA vectors present, as analyzed by polymerase chain reaction (PCR) and ADA activity assay (Fig. 3). The expected 415-bp band was amplified by PCR with Neo-specific primers (Fig. 3A), and its specificity confirmed by control hybridization to a Neo radioactive probe (Fig. 3B). All PCR-positive clones had ADA activity comparable to or higher than that of normal controls (Fig. 3C). A number of these clones were expanded and DNA analyzed by Southern blot for integration of proviral sequences (*17*). Major integration bands of different sizes were observed in the majority of clones, indicating their essentially clonal nature. However, integration bands with apparently the same molecular weight were observed in three independent clones, suggesting that at least

Table 1. BNX mice reconstituted with DCA-infected PBLs obtained from ADA-deficient patients. Peripheral blood lymphocytes were obtained from two different ADA$^-$ SCID patients (G.B. and A.H.) and six adult normal controls and utilized to reconstitute 32 BNX mice as in (*12*). N.D., not done.

Donor	DCA vector infection	Mouse number	Human-IgG serum levels (mg/dl)		In recipient spleen			Number of clones from recipient spleen		
			2 weeks	8 weeks	CD4$^+$ (%)	hu-ADA	Alu	CD4$^+$	DCA$^+$	hu-ADA
ADA positive controls (n = 20)	-		6.3* (0.5–43.8)	24.6* (0.5–429.0)	5.4* (0–12.1)	4/7†	5/11†	3/5†	N.D.	N.D.
G.B.	+	711	N.D.	20.0	N.D.	–	–	N.D.	N.D.	N.D.
G.B.	+	714	N.D.	48.0	1.6	+	+	N.D.	N.D.	N.D.
G.B.	+	727	N.D.	375.5	3.9	++	+	>33	33	33
G.B.	+	751	54.0	672.1	4.2	+	+	58	57/58	57/58
G.B.	+	757	8.4	29.8	1.4	N.D.	–	0	N.D.	N.D.
G.B.	+	621	0.5	0.5	1.0	–	–	0	N.D.	N.D.
A.H.	+	651	1.1	3.7	2.1	±	±	5	5	5
A.H.	+	721	8.0	44.1	1.2	N.D.	N.D.	N.D.	N.D.	N.D.
G.B.	–	715	8.4	1.1	0.0	–	–	0	N.D.	N.D.
G.B.	–	716	2.0	0.7	0.2	–	–	0	N.D.	N.D.
G.B.	–	731	0.5	0.5	0.0	–	–	0	N.D.	N.D.
A.H.	–	644	0.5	0.5	0.0	–	–	N.D.	N.D.	N.D.

*Median with range in parenthesis. †Positive/tested recipient animals.

in some cases T cells may have originated from common progenitors.

We were unable to establish Epstein-Barr virus (EBV)–transformed B lymphoblastoid continuous cell lines (*18*) from animals reconstituted with DCA-infected ADA$^-$ PBLs. Whether this was the result of a lack of DCA vector infection of ADA$^-$ B lymphocytes or refractoriness of vector transduced B cells to EBV transformation could not be determined. However, it was also difficult to obtain EBV-transformed cell lines from PBLs freshly isolated from the patient, which may correlate with the low expression of C3d, the EBV receptor, on the B cells of this patient.

We also tested the capacity of vector-transduced ADA$^-$ PBLs to express antigen-specific immune responses in our in vivo model. Vector-infected ADA$^-$ PBLs used in the experiment were from the patient undergoing enzyme-replacement treatment with PEG-ADA (*12*). This patient had been vaccinated with tetanus toxoid (tt) and produced specific antibodies and specific T cells that proliferated in vitro. A mouse reconstituted with these cells (*727*) was immunized with tt plus tt-pulsed irradiated PBLs from the same patient (as antigen presenting cells). Two weeks later, spleen cells were obtained and restimulated in vitro with autologous, tt-pulsed irradiated PBLs (*19*). In this experiment limiting dilution was not performed because of the limited availability of the patient PBLs as feeder cells. T cell lines (30 of 33 tested) proliferated specifically to tt-pulsed but not to unpulsed antigen-presenting cells and had large amounts of vector-derived ADA activity. The capacity of the transduced human cells to produce specific immune responses was confirmed by the increased production of antigen-specific hu-IgG (*20*). In animal *727*, circulating tt-specific hu-IgG significantly increased 2 weeks after in vivo restimulation with tt and tt-pulsed PBLs. A summary of all treated animals is shown in Table 1.

In the in vivo model described here, we obtained evidence that a gene therapy approach to ADA$^-$ SCID could be potentially successful. ADA$^-$ T cells transduced with an ADA-expressing retroviral vector survived relatively long in vivo. Proliferation and expansion of human lymphocytes in recipient animals could not be formally demonstrated, although independent clones sharing common integration sites were obtained from at least one reconstituted animal. Correction of the ADA deficiency by expression of a functional, vector-derived enzyme appears to restore normal T cell functions, including antigen-specific immune responses. In this regard, previous studies on the clinical efficacy of PEG-ADA treatment have demonstrated that detoxification of the extracellular environment may lead to reconstitution of the lymphocyte populations and of several immune functions (*12*). However, we show that synthesis of vector-derived enzyme confers to the positively transduced lymphocytes a significant advantage over non-transduced cells in vivo, regardless of the presence of a detoxifying environment.

If this strategy is used in the clinic (*21*), the procedure may have to be repeated periodically. However, the chimeric status of nonconditioned ADA$^-$ SCID patients treated with allogeneic bone marrow transplantation indicates that long-term immune reconstitution can be provided by donor T cells, whereas all other cell lineages, including B lymphocytes, are of host origin (*18*). Thus, the long-term reconstitution of T cells and T cell functions could come from either marrow or peripheral blood lymphocyte precursors.

Bone marrow cells and peripheral blood lymphocytes from ADA-deficient SCID patients cannot respond to nonspecific proliferative stimuli such as IL-2 and PHA or to specific antigens. The restoration of PHA and IL-2 responses in vitro and production of antigen-specific antibodies and T cells in immunodeficient mice reconstituted with DCA-infected cells in vivo give physiologic relevance to this gene transfer system. For patients failing therapy with PEG-ADA, the gene therapy approach may represent a safe and efficacious alternative.

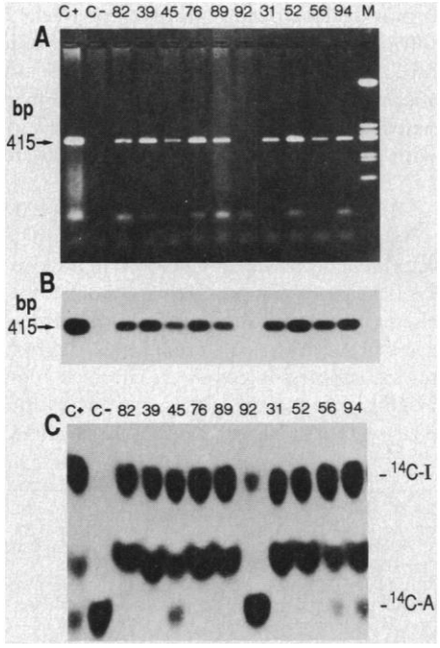

Fig. 3. Detection of DCA retroviral genomes and analysis of ADA activity in human T cell clones from BNX mouse spleen 751. (**A**) PCR analysis. A retroviral-specific 415-bp fragment located in the coding region of the neomycin phosphotranspherase gene (Neo^R) was amplified from 1 μg of genomic DNA by 30 cycles of PCR with 5 units of Taq polymerase and 100 pmol of the oligonucleotide primers Neo-1 (5′-GGAAGCCGGTCTTGTCGATC-3′) and Neo-2 (5′-CGAAATCTCGTGATGGCAGG-3′), using a commercially available reagent kit and a cyclic reactor from Perkin-Elmer Cetus. The reaction mixture (1/10 of the total volume; 100 μl) was separated on a 1.6% agarose gel, and DNA visualized by ethidium bromide staining. The molecular weight marker (M) is a Pst I and Dde I digest of pBR322 (International Biotechnologies, Inc.). Positive control (C$^+$) is DNA from Raji (human Burkitt's lymphoma) cells infected with DCA and selected in G418. Negative control (C$^-$) is normal PBL DNA. (**B**) Control hybridization of PCR products. DNAs were transferred from the agarose gel to a nylon membrane (Hybond-N, Amersham) by Southern blotting, and hybridized to 10^7 dpm of ^{32}P-labeled, 1.2-kb Hind III–Sma I fragment of pSV2-Neo (*22*). The filter was washed under high stringency conditions and exposed to a Kodax X-AR5 film for 30 min at −70°C. (**C**) Thin-layer chromatography analysis of ADA activity. ADA enzyme activity was analyzed by the [^{14}C]adenosine (^{14}C-A) to [^{14}C]inosine (^{14}C-I) conversion assay (*23*). Cell lysates from individual clones (~2 × 10^5 cells) were normalized for protein content. Cell lysates from normal PBLs and uninfected ADA$^-$ PBLs were used as positive (C$^+$) and negative (C$^-$) controls, respectively. Because IL-2 stimulation may increase the efficiency of ADA expression in ADA deficient cells (*24*), ADA-deficient IL-2–stimulated cells were used as negative controls in the reported experiments.

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12. Freshly isolated ADA$^-$ PBLs were obtained from two ADA$^-$ SCID patients. The diagnosis was confirmed by immunologic tests and assay of ADA

activity in peripheral blood mononuclear cells and erythrocytes, which ranged between 0.2 and 1.1% of normal controls. One of the patients (G.B.) was being treated with polyethylene glycol–conjugated enzyme (PEG-ADA, Enzon, South Plainfield, NJ) replacement therapy [M. S. Hershfield *et al.*, *N. Engl. J. Med.* **316**, 589 (1987); Y. Levy *et al.*, *J. Ped.* **113**, 312 (1988); C. Bordignon *et al.*, in preparation] and had normal lymphocyte counts. Under parental informed consent, ADA^- PBLs were obtained by Ficoll-Hypaque separation and subjected to multiple infection cycles with cell-free viral stock in the presence of Polybrene (4 μg/ml), at high vector to cell ratio (2 to 5 colony-forming units per cell) under phytohemagglutinin (PHA) and interleukin-2 (IL-2) stimulation (2 μl of purified PHA, Wellcome Laboratories, Dartford, U.K.; 100 U of human recombinant IL-2, Roche, Nutley, NJ). After completion of the multiple infection cycles, PBLs were resuspended in phosphate-buffered saline and injected intraperitoneally into mice (2×10^7 to 5×10^7 cells per mouse). Recipient BNX mice received no additional cytoreduction or immunosuppression prior to reconstitution with human cells.

13. Human immunoglobulins in the serum of BNX mice was measured with an automated Behring nephelometer analyzer that relies on a specific rabbit antibody to hu-IgG (Behringwerke, Marburg, Germany).
14. High molecular weight DNA was obtained from 1 $\times 10^7$ 3 $\times 10^7$ to spleen cells by standard phenol-chloroform extraction [J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, ed. 2, 1989)], denaturated dot blotted on nylon membranes [S. J. Collins and M. T. Groudine, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4831 (1983)], and hybridized to 10^7 dpm of a 1.8-kb Eco RI–Bam HI fragment containing the human ε-globin Alu repeat [C. Di Segni, G. Carrara, G. R. Tocchini-Valentini, C. C. Shoulders, F. E. Baralle, *Nucleic Acids Res.* **9**, 6709 (1981)], labeled by random priming to a specific activity of 10^9 dpm/μg.
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16. Spleen cells from BNX reconstituted mice were cultured in Terasaki microplates with RPMI 1640 supplemented with 2 mM L-glutamine, 1% nonessential amino acids, 1% Na pyruvate, kanamycin (50 mg/ml) (complete RPMI), 5% human serum (HS) and human recombinant IL-2 (hu-rIL-2) (100 U/ml) (Roche, Nutley, NJ) under limiting dilution conditions. The cell concentrations used (10^4, 10^3, 10^2, and 10 cells per well) were chosen according to the estimated frequencies of human cells in the spleens. After 1 to 2 weeks the positive wells were scored and transferred to 96-well flat-bottom plates. The clones were maintained in complete RPMI medium supplemented with 5% HS and hu-rIL-2 (100 U/ml) and restimulated every 2 to 3 weeks with irradiated (3000 R) allogeneic PBLs.
17. Ten micrograms of high molecular weight DNA were cut to completion with Xba I, which cuts twice in the DCA provirus, or Hind III, which cuts only in cellular DNA. Digests were separated on a 0.8% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham), hybridized to 10^7 dpm of the Neo^R-specific probe described in the legend to Fig. 3, washed at high stringency, and exposed to Kodak X-AR5 films for 1 to 4 days.
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19. Tetanus toxoid (tt) (50 μg/ml; Swiss Serum Institute, Bern, Switzerland) together with 5×10^5 tt-pulsed irradiated autologous PBLs as antigen presenting cells were injected intraperitoneally into a recipient BNX mouse 6 weeks after injection with DCA-infected ADA^- PBLs. Two weeks later, spleen cells were obtained and 10^5 cells per well were stimulated in 96-well flat-bottom plates in complete RPMI medium supplemented with 5% HS, with 5×10^5 tt-pulsed (20 μg/ml) irradiated (3000 R) autologous PBLs per well. The growing T cell lines were maintained in complete RPMI medium, 5% HS and hu-rIL-2 (100 U/ml), and restimulated every 2 to 3 weeks with 10^6 irradiated autologous PBLs and 20 μg/ml of tetanus toxoid. For T cell stimulation, autologous PBLs were either left untreated or pulsed overnight with 20 μg/ml of tt. T cells (4×10^4) were cultured with 10^5 irradiated PBLs. After 48 hours the cultures were pulsed with 1 μCi per well of [^{3}H]thymidine (Amersham, specific activity 5 Ci/mM) and the radioactivity incorporated was measured after an additional 16 hours. Thin-layer chromatography (TLC) analysis of ADA activity in tt-specific clones was done as in Fig. 3.
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Identification of a Gene Located at Chromosome 5q21 That is Mutated in Colorectal Cancers

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Recent studies have suggested the existence of a tumor suppressor gene located at chromosome region 5q21. DNA probes from this region were used to study a panel of sporadic colorectal carcinomas. One of these probes, cosmid 5.71, detected a somatically rearranged restriction fragment in the DNA from a single tumor. Further analysis of the 5.71 cosmid revealed two regions that were highly conserved in rodent DNA. These sequences were used to identify a gene, MCC (*m*utated in *c*olorectal *c*ancer), which encodes an 829–amino acid protein with a short region of similarity to the G protein–coupled m3 muscarinic acetylcholine receptor. The rearrangement in the tumor disrupted the coding region of the MCC gene. Moreover, two colorectal tumors were found with somatically acquired point mutations in MCC that resulted in amino acid substitutions. MCC is thus a candidate for the putative colorectal tumor suppressor gene located at 5q21. Further studies will be required to determine whether the gene is mutated in other sporadic tumors or in the germ line of patients with an inherited predisposition to colonic tumorigenesis.

STUDIES OVER THE PAST SEVERAL years have allowed formulation of a genetic model for colorectal tumorigenesis (*1*). It appears that accumulated alternations of at least one proto-oncogene (often K-RAS on chromosome 12) and of several suppressor genes (on chromosomes including 5, 17, and 18) are required for malignant tumor formation; fewer changes suffice for benign tumorigenesis. To date, candidate colorectal tumor suppressor genes have been identified on chromosome 17p (*2*) and 18q (*3*). Cytogenetic (*4*) and linkage (*5, 6*) studies have shown that chromosome region 5q21 harbors the gene responsible for familial adenomatous polyposis (FAP), an autosomal-dominant, inherited disease in which affected individuals develop hundreds to thousands of adenomatous polyps, some of which progress to malignancy. Additionally, this chromosomal region is often deleted from the adenomas (*7*) and carcinomas (*7–11*) of patients without FAP. Thus, a putative suppressor gene at 5q21 appears to be involved in the early stages of colorectal neoplasia in both sporadic and familial tumors.

The idea that the same gene on 5q may be mutated somatically in sporadic tumors and mutated in the germ line of FAP patients is consistent with the hypothesis formulated

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