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 1×10^7 3×10^7 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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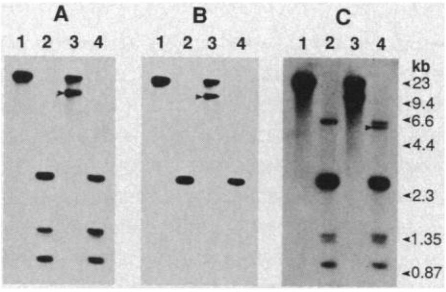

Fig. 1. Southern blot analysis of tumor T14 demonstrating a somatic change. Lanes 1 and 2 contain 5 μg of DNA isolated from normal tissue of patient T14; lanes 3 and 4 contain 5 μg of DNA isolated from the T14 colon carcinoma. In lanes 1 and 3, DNA was cleaved with Eco RI; in lanes 2 and 4, DNA was cleaved with Pst I. The Southern blot in panel (**A**) was hybridized to a subclone of cosmid 5.71 (5.71-3). Panels (**B**) (3-hour exposure) and (**C**) (20-hour exposure) show the same Southern blot hybridized with the abnormal 11-kb fragment cloned from the T14 tumor. The arrowheads indicate the novel alterations in T14. Southern blot analysis was as described (*18*), except that hybridizations were at 60°C and washes were at 65°C. The size markers indicated on the right represent Hind III–cleaved λ DNA and Hae III–cleaved ϕX phage DNA.

by Knudson for the action of tumor suppressor genes (*12*). Therefore, two interconnected approaches were used in this study. In the first approach, FAP was treated as a standard inherited disease and linkage analysis was used to define a small region of chromosome 5q21 containing the relevant gene. In the second approach, allelic losses of this region in sporadic tumors complemented the linkage analysis and a putative suppressor gene was identified through a search for somatically acquired mutations.

Numerous clones detecting restriction fragment length polymorphisms (RFLPs) have been derived from 5q21 (*13, 14*). Several of these RFLP markers are tightly linked to disease phenotype in FAP kindreds, with lod scores in excess of 8.0 and recombination fractions of less than 0.01. When these and flanking RFLP markers were used, allelic loss was found in more than 30% of sporadic colorectal carcinomas analyzed (*7–11*). The region of common loss appeared to be centered at the RFLP detected by cosmid 5.71 (*14*). Portions of cosmid 5.71 were subcloned and used as probes to screen a panel of 150 colorectal carcinomas by Southern (DNA) blot analysis. We found one tumor (T14) that contained an 11-kb Eco RI fragment in addition to the 20-kb Eco RI fragment seen in DNA from normal individuals. The 11-kb fragment was not present in DNA isolated from normal cells from the same patient (Fig. 1A). The new Eco RI fragment was cloned (*15*), and used to probe Southern blots with DNA from tumor T14. The 11-kb clone hybridized to the abnormal 11-kb Eco RI fragment and to the normal 20-kb Eco RI fragment in the tumor as expected (Fig. 1B). Moreover, the 11-kb clone detected new fragments in tumor T14 DNA upon digestion with other restriction endonucleases [including Pst I (Fig. 1C), Hind III, and Eco RV (*14*)]. Restriction mapping and partial sequencing of the 11-kb clone showed that its left end was derived from the 20-kb Eco RI fragment that contained cosmid 5.71 sequences. The right end of the 11-kb fragment was derived from sequences that were not contiguous with the left end in normal genomic DNA. Use of a 400-bp probe from the right end of the 11-kb fragment showed that the noncontiguous sequences were also derived from chromosome 5, but from a position separated by at least 100 kb from the left end of the 11-kb Eco RI fragment. Thus a rearrangement had occurred in the tumor that resulted in the juxtaposition of sequences that were normally far apart.

We assumed that this rearrangement affected a gene near the sequences in cosmid 5.71. To find the gene, we used a cross-hybridization strategy, based on the fact that human DNA sequences that cross-hybridize to the DNA of other mammalian species often correspond to expressed genes (*3, 16, 17*). We identified two subclones (5.71-5 and 5.71-3) that cross-hybridized to rodent DNA under conditions of reduced stringency (*18*). However, attempts to use these sequences to detect expressed genes by Northern (RNA) blotting and cDNA library screening of more than 3×10^6 colon or brain cDNA clones were unsuccessful.

In order to increase the sensitivity of our expression assay, we turned to the "exon-connection" strategy (*3*), which detects genes expressed at low levels. In brief, primers are derived from two putative exons of the gene in question. Amplification by the polymerase chain reaction (PCR) was done with these primers and cDNA as template. This procedure allows detection of the putative exons if they are joined by RNA splicing within cells. Contaminating genomic DNA in the RNA preparation does not interfere with this assay, since the intervening intron (or introns) results in much longer PCR products from genomic DNA than that obtained from the spliced RNA.

To initiate this process, we sequenced parts of the human subclones that had shown cross-species hybridization, but found it impossible to predict exons from this sequence information alone. Through comparison of the sequences of the corresponding rat and human regions (*19*) one putative exon from 5.71-3 and one from 5.71-5 were identified (Fig. 2). Each contained an open reading frame (ORF) that was preceded and followed by splice acceptor and donor sites that were conserved between species. The predicted ORFs from the rat and human exons were 96% identical at the amino acid level and 89% identical at the nucleotide level, with most of the nucleotide differences occurring at the third position of codons.

The exon-connection strategy was then applied to these two putative exons, which are separated in DNA by more than 2 kb. We did not initially know the orientation of the putative exons with respect to one another and therefore designed two sets of primers (Fig. 2). One set (primers P1 and P4) would have resulted in a PCR product if the exon in 5.71-5 was upstream of that in 5.71-3. The other set (primers P2 and P3) would have allowed detection of a PCR product if the exons were in the reverse orientation. We found that only primers P1 and P4 resulted in a PCR product when cDNA was used as template (*20*). This PCR product was detected with cDNA derived

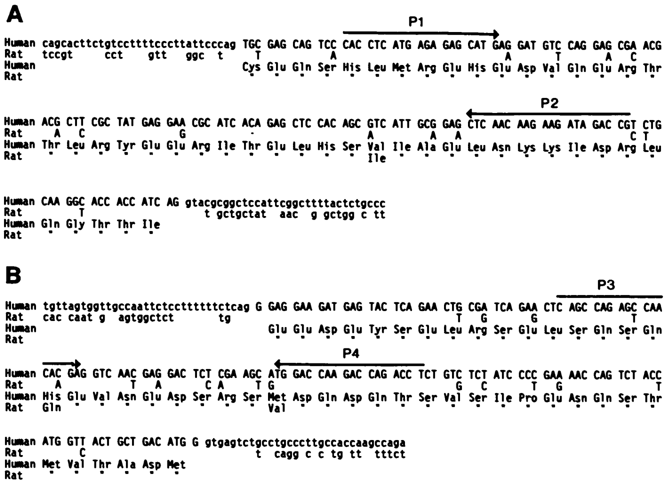

Fig. 2. Sequence of putative exons from the 5.71 cosmid. (**A**) shows the sequence of the 5.71-5 exon and the related rat exon. (**B**) shows the sequence of the 5.71-3 exon and the related rat exon. Rat sequences are listed only where they differ from the human sequence. Lower case letters signify introns surrounding the exons. The primers used for PCR are demarcated by arrows. Primers P2 and P4 were reversed and complemented relative to the sequence shown. Rat clones were obtained and sequenced as described (*19*).

A

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                                                                  P1
                                                      ------------------------>
Human cagcacttctgtccttttcccttattcccag TGC GAG CAG TCC CAC CTC ATG AGA GAG CAT GAG GAT GTC CAG GAG CGA ACG
Rat   tccgt     cct    gtt   ggc  t     T           A                           A       T       A   C
Human                                 Cys Glu Gln Ser His Leu Met Arg Glu His Glu Asp Val Gln Glu Arg Thr
Rat                                    .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

                                                                                       P2
                                                                          <--------------------------
Human ACG CTT CGC TAT GAG GAA CGC ATC ACA GAG CTC CAC AGC GTC ATT GCG GAG CTC AAC AAG AAG ATA GAC CGT CTG
Rat     A   C               G          -                  A         A   A                           C T
Human Thr Leu Arg Tyr Glu Glu Arg Ile Thr Glu Leu His Ser Val Ile Ala Glu Leu Asn Lys Lys Ile Asp Arg Leu
Rat    .   .   .   .   .   .   .   .   .   .   .   .   .  Ile  .   .   .   .   .   .   .   .   .   .   .

Human CAA GGC ACC ACC ATC AG gtacgcggctccattcggctttactctgccc
Rat         T                   t gctgctat  aac g gctgg c tt
Human Gln Gly Thr Thr Ile
Rat    .   .   .   .   .
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B

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                                                                                                  P3
                                                                                       -------------------
Human tgttagtggttgccaattctcctttttttctcag G GAG GAA GAT GAG TAC TCA GAA CTG CGA TCA GAA CTC AGC CAG AGC CAA
Rat   cac caat g  agtggctct        tg                                    T   G       G               T
Human                                      Glu Glu Asp Glu Tyr Ser Glu Leu Arg Ser Glu Leu Ser Gln Ser Gln
Rat                                         .   .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

                                                       P4
      ------>                              <------------------------
Human CAC GAG GTC AAC GAG GAC TCT CGA AGC ATG GAC CAA GAC CAG ACC TCT GTC TCT ATC CCC GAA AAC CAG TCT ACC
Rat     A           T   A       C A     T G                             G   C       T   G               T
Human His Glu Val Asn Glu Asp Ser Arg Ser Met Asp Gln Asp Gln Thr Ser Val Ser Ile Pro Glu Asn Gln Ser Thr
Rat   Gln  .   .   .   .   .   .   .   .  Val  .   .   .   .   .   .   .   .   .   .   .   .   .   .   .

Human ATG GTT ACT GCT GAC ATG G gtgagtctgcctgcccttgccaccaagccaga
Rat         C                           t  cagg c c tg tt  tttct
Human Met Val Thr Ala Asp Met
Rat    .   .   .   .   .   .
```

from mRNA of normal human colon. The PCR product was exactly the size (226 bp) expected if direct splicing of the two putative exons had occurred at the splice sites identified in the human and rat genomic DNA sequences. Cloning and sequencing of the PCR product confirmed that it represented the result of a direct splice between the 5.71-5 and 5.71-3 exons. This spliced product produced an in frame fusion of the ORFs from each exon. We concluded that these sequences did indeed represent an expressed gene, hereinafter referred to as the MCC gene for *m*utated in *c*olorectal *c*ancer.

By means of the exon-connection strategy, we found that MCC was expressed in most normal tissues of the rat (such as colon, brain, stomach, lung, liver, kidney, bladder, and heart) (*14*). The PCR product was then labeled and used as a probe to screen a cDNA library from normal human brain (*21*). Brain was chosen because the exon-connection assay suggested that MCC was expressed at high levels in this tissue. Three clones were identified in 1.5 × 10^6 plaques in the initial screen. The ends of these three clones were then used to rescreen the library, and seven overlapping cDNA clones were finally isolated and ordered. Sequence analysis of these clones indicated that they encompassed 4181 bp of MCC mRNA and contained an ORF of 2511 bp (Fig. 3A). The first methionine of the ORF (nucleotide 221) was preceded by in frame stop codons upstream and conformed reasonably well to the consensus initiation site defined by Kozak (*22*). If translation initiation occurs at this methionine, the sequence predicts an 829–amino acid product (93 kD) encoded from nucleotide 221 to 2708. The ORF was surrounded by at least 200 bp of 5′ untranslated sequence and 1450 bp of 3′ untranslated sequence. There was no evidence of a polyadenylation tract at the 3′ end of any clone. The cDNA probes detected RNAs of several sizes (5 to 10 kb) on Northern blots (*14*); we do not know whether these other transcripts represent alternatively spliced forms of the MCC gene or related genes from other loci.

Fig. 3. (**A**) Predicted amino acid sequence of MCC. The nucleotide and amino acid sequences have been deposited at Genbank (accession number M62397). (**B**) Comparison of MCC and the G protein–activating region of human m3 muscarinic acetylcholine receptor (*27*). Connecting lines indicate identities; dots indicate related amino acid residues. (A) refers to the 10–amino acid region which, when deleted, alters G protein responses. (B) refers to the nine amino acids that can mediate specificity of mAChR G protein coupling.

A

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MNSGVAMKYG NDSSAELSEL HSAALASLKG DIVELNKRLQ QTERERDLLE KKLAKAQCEQ  60
SHLMREHEDV QERTTLRYEE RITELHSVIA ELNKKIDRLQ GTTIREEDEY SELRSELSQS 120
QHEVNEDSRS MDQDQTSVSI PENQSTMVTA DMDNCSDLNS ELQRVLTGLE NVVCGRKKSS 180
CSLSVAEVDR HIEQLTTASE HCDLAIKTVE EIEGVLGRDL YPNLAEERSR WEKELAGLRE 240
ENESLTAMLC SKEEELNRTK ATMNAIREER DRLRRRVREL QTRLQSVQAT GPSSPGRLTS 300
TNRPINPSTG ELSTSSSSND IPIAKIAERV KLSKTRSESS SSDRPVLGSE ISSIGVSSSV 360
AEHLAHSLQD CSNIQEIFQT LYSHGSAISE SKIREFEVET ERLNSRIEHL KSQNDLLTIT 420
LEECKSNAER MSMLVGKYES NATALRLALQ YSEQCIEAYE LLLALAESEQ SLILGQFRAA 480
GVGSSPGDQS GDENITQMLK RAHDCRKTAE NAAKALLMKL DGSCGGAFAV AGCSVQPWES 540
LSSNSHTSTT SSTASSCDTE FTKEDEQRLK DYIQQLKNDR AAVKLTMLEL ESIHIDPLSY 600
DVKPRGDSQR LDLENAVLMQ ELMAMKEEMA ELKAQLYLLE KEKKALELKL STREAQEQAY 660
LVHIEHLKSE VEEQKEQRMR SLSSTSSGSK DKPGKECADA ASPALSLAEL RTTCSENELA 720
AEFTNAIRRE KKLKARVQEL VSALERLTKS SEIRHQQSAE FVNDLKRANS NLVAAYEKAK 780
KKHQNKLKKL ESQMMAMVER HETQVRMLKQ RIALLEEENS RPHTNETSL             829
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B

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MCC       220 LYPNLAEERSRWEKELAGLREENE 243
              ||  :  |  :  ||||||
m3 mAChR  249 LYWRIYKETEKRTKELAGLQASGT 272
DOMAIN A           YKETEKRTKE
DOMAIN B                     LAGLQASGT
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Searches of nucleotide databases (European Molecular Biology Library version 25, Genbank version 66) indicated that this sequence has not been previously reported. Searches of amino acid database (Protein Identification Resource version 25, SWISS-Protein version 16) with the predicted MCC protein (829 amino acids) also did not reveal any extensive similarities. However, we noted a 19–amino acid region of similarity between MCC and the G protein–coupled m3 muscarinic acetylcholine receptor (mAChR) of humans and pigs (Fig. 3B); the COOH-terminal six amino acids (KELAGL) were identical (Fig. 3B). Initially, we did not know whether this similarity was significant, because many other proteins had higher levels of global similarity (though few had six contiguous amino acids in common). During our search for mutations, however, a study on the sequence elements controlling G protein activation by mAChR subtypes was published (*27*). It was shown that a 21–amino acid region from the m3 mAChR completely mediated G protein specificity when substituted for the 21 amino acids of m2 mAChR at the analogous protein position. These 21 residues overlapped the 19–amino acid region of similarity between MCC and m3 mAChR (Fig. 3B). A ten-residue deletion (Fig. 3B, domain A), which included the two NH_2-terminal amino acids of the KELAGL motif, completely altered the kinetics and magnitude of the G protein–mediated response. Moreover, a nine-residue subdomain (Fig. 3B, domain B) that included the four COOH-terminal amino acids of KELAGL was sufficient for specifying the activation of the m3 G protein pathway when transferred to the m2 mAChR.

When the sequences of MCC were compared with those of genomic clones from tumor T14 it was found that the boundary of the rearrangement in this tumor was within MCC, occurring in the intron just distal to the exon encoding amino acids 105 to 152. As noted above, the novel 11-kb restriction fragment represented the joining of sequences on chromosome 5 that are normally separated by more than 100 kb. This 100-kb stretch contained several exons of the MCC gene. Thus, the MCC gene was disrupted by a genetic alteration that removed several exons from the rearranged MCC gene in this tumor.

To search for other more subtle genetic alterations of MCC, we used PCR to amplify exons of MCC from colorectal cancers. These sequences were then analyzed for mutations by a ribonuclease (RNase) protection assay that was modified to allow rapid testing of multiple samples. The sequence of an exon and surrounding intron was determined and used to design primers for the amplification of the exon and surrounding splice sites from tumor DNA (*23*). The resulting PCR products were hybridized to in vitro–generated RNA probes representing normal MCC sequences. The hybrids were digested with RNase A, which can cleave at single base pair mismatches within DNA-RNA hybrids, and these cleavage products were visualized after denaturing gel electrophoresis (*24*). Two separate RNase protection analyses were done for each exon, one with the sense and one with the antisense strand as labeled transcript. Under these conditions approximately 50% of all point mutations are detectable (*14*, *25*).

The first exon (encoding amino acids 58 to 104) of four tested showed no variants among 100 colorectal tumors tested. Analysis of a second exon, encoding amino acids 453 to 486, identified five tumors with identical variations in their RNase protection pattern. Cloning and sequencing of the variant PCR product from two of the five tumors indicated that it resulted from a C to T transition in codon 486 that resulted in a change from proline to leucine. This variant presumably represents a polymorphism, as it was found in five individuals and was present in DNA from the normal tissue of two of the five patients whose tumors showed the variant (the other three were not tested).

Analysis of a third exon (encoding amino acids 696 to 729) identified a single tumor (T91), which had an abnormal RNase protection pattern that was not seen in DNA isolated from normal tissue from the same individual (Fig. 4A). This indicates that the altered RNase protection pattern was the result of a somatic mutation. Cloning and sequencing of the T91 tumor PCR product indicated that it had a C to T transition in codon 698 that resulted in a change from alanine to valine. Although this is a relatively conservative amino acid substitution, the identical amino acid change has been shown to inactivate the p53 tumor suppressor gene (*2, 26*).

Analysis of a fourth exon (encoding amino acids 487 to 548) identified a single tumor (T35) with a variant RNase protection pattern. Examination of DNA isolated from normal tissue of the same individual indicated that this pattern was also the result of a somatic mutation (Fig. 4B). Cloning and sequencing of the T35 PCR product indicated that it had a G to A transition in codon 506 resulting in a coding change from arginine to glutamine.

In summary, the results described above have allowed definition of a previously undescribed gene located at 5q21. The gene was found to be the target of somatic mutations in at least three tumors, one in which it was disrupted by a rearrangement/deletion and two in which it was altered by single base pair mutations. The MCC gene is therefore a candidate for the suppressor gene on 5q21 inferred from previous studies.

The connection between MCC and the G protein–activating region of mAChR is intriguing in light of previous investigations relating G proteins to cancer. For example, the RAS oncogenes, which are often mutated in colorectal cancers (*7, 28*), are members of the G protein family (*29*) as is an in vitro transformation suppressor (*30*) and genes mutated in hormone-producing tumors (*31*). Additionally, the gene responsible for neurofibromatosis I (presumably a tumor suppressor gene) has been shown to activate the guanosine triphosphatase activity of RAS (*32*). Another link between G proteins and colon cancer involves the drug sulindac. This agent has been shown to inhibit the growth of benign colon tumors in patients with FAP, presumably by virtue of its activity as a cyclooxygenase inhibitor (*33*). Cyclooxygenase is required to convert arachidonic acid to prostaglandins and other biologically active molecules. G proteins are known to regulate phospholipase A2 activity, which generates arachidonic acid from phospholipids (*34*). It will be of interest to determine if MCC interacts with a G protein and whether such a G protein regulates phospholipase A2 activity.

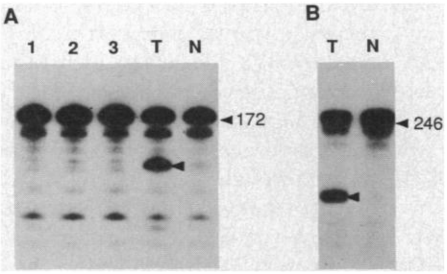

Fig. 4. PCR–RNase protection analysis. RNase protection analysis was performed on PCR products as described (*24*) and the resulting cleavage products separated by denaturing gel electrophoresis. Amino acids 696 to 729. (**A**) shows the results of analysis of the exon encoding amino acids 696 to 729. Lanes 1, 2 and 3 show the results obtained from DNA isolated from three tumors that did not show any changes. Lanes marked T and N show the results obtained from DNA isolated from patient 91's tumor or normal cells, respectively. Panel (**B**) shows the results of analysis of the exon encoding amino acids 487 to 548. Lanes marked T and N show the results obtained from DNA isolated from patient 35's tumor and normal cells, respectively. Arrowheads indicate novel RNase protection products. Sizes of full-length products are indicated in base pairs.

It should be emphasized that the studies described above do not prove that the MCC gene is the tumor suppressor gene in this region. The rearrangement in tumor T14 clearly disrupted the MCC gene by removing multiple exons. However, this rearrangement may have resulted in the disruption of other genes close to MCC and one of these other genes could represent the true target of somatic alteration in this tumor. The two point mutations provide more definitive evidence for changes that exclusively affect a single gene in this region. Such somatic mutations have previously been observed only in oncogenes and tumor suppressor genes (*35*). However, it is theoretically possible that the two MCC point mutations observed were without biological effect and had simply become "fixed" in the tumor cell population by virtue of another mutation that coincidentally occurred in the same cell and provided a selective growth advantage. For these reasons, additional studies will be required to elucidate the relationship between MCC gene mutations and sporadic colorectal cancers. It will also be of interest to determine whether MCC is mutated in the germ line of FAP patients.

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19. A rat genomic library in the lambda DASH vector (Stratagene) was probed with ^{32}P-labeled 5.71-3 and 5.71-5 sequences. Cross-hybridizing restriction fragments of these phage clones were subcloned into plasmid vectors and sequenced to derive the similarities shown in Fig. 2. Sequencing was performed with unmodified T7 polymerase as described by G. Del Sal, G. Manfioletti, and C. Schneider [*BioTechniques* 7, 514 (1989)].
20. PCR was performed as described in S. Baker *et al.* [*Cancer Res.* **50**, 7717 (1990)] with 35 cycles of the following: 95°C for 0.5 min, 55°C for 2 min, and 70°C for 2 min.
21. The cDNA library was constructed from human brain mRNA as described by U. Gubler and B. J. Hoffman [*Gene* **25**, 263 (1983)] and the Lambda Zap vector (Stratagene). Plaques (1.5 × 10^6) were screened with the PCR product connecting the 5.71-3 and 5.71-5 exons (Fig. 2). Three clones were initially identified and used to rescreen the library. Seven overlapping cDNA clones were finally isolated and their composite sequence is shown in Fig. 3. Sequencing was performed on both strands (*19*) and was verified in at least two independent clones for each nucleotide position.
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and 0.3% w/v bromophenol blue in formamide). The samples were then heated at 94°C for 4 min and loaded directly on a denaturing polyacrylamide gel. We performed two separate assays for each exon using each strand as a labeled transcript.
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Technical Comments

Sodium-Calcium Exchange

N. Leblanc and J. R. Hume (*1*) conclude that the intracellular calcium ion concentration ($[Ca^{2+}]_i$) of cardiac myocytes can increase transiently, in the absence of an inward Ca^{2+} current, by the influx of Ca^{2+} through Na^+-Ca^{2+} exchange, this influx being induced primarily by the influx of Na^+ through tetrodotoxin (TTX)-sensitive Na^+ channels. We suggest an alternative explanation.

Leblanc and Hume base their conclusion on their observations that TTX reduces the size of the transient rise in $[Ca^{2+}]_i$ in response to an action potential and that, in voltage-clamp experiments, inactivation of the fast Na^+ current by a conditioning depolarization reduces the size of the transient rise in $[Ca^{2+}]_i$ in response to a subsequent depolarization. Moreover, in cells exposed to the putative Ca^{2+} channel blocker, dihydropyridine, a depolarization-induced (TTX-sensitive) Na^+ current is associated with a transient rise in $[Ca^{2+}]_i$ *only* in the presence of extracellular Ca^{2+}.

Leblanc and Hume suppose that the TTX-sensitive rise in $[Ca^{2+}]_i$ arises as a result of an influx of Na^+ through TTX-sensitive Na^+ channels, which causes a transient rise in intracellular Na^+ concentration ($[Na^+]_i$) near the inner surface of the sarcolemma that shifts the reversal potential of the Na^+-Ca^{2+} exchanger toward negative membrane potentials. This shift in reversal potential would thereby promote a transient influx of Ca^{2+} through the exchanger that, in turn, triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR).

As pointed out by W. J. Lederer *et al.* (*2*), the idea of a TTX-sensitive Na^+ current as the primary event poses a problem. Unless the volume into which the entering Na^+ is distributed is restricted (at least in the short term) to a small fraction of the total cell volume, the rise in $[Na^+]_i$ would have an insignificant effect on the reversal potential of the exchanger. Even allowing for the fact that the rise in $[Na^+]_i$ need not be as high as that presumed by Lederer *et al.* (8 mM) to cause the necessary rise in $[Ca^{2+}]_i$ to trigger Ca^{2+} release from the SR (*3*), there is no escaping the need for some restriction in the space into which the incoming Na^+ diffuses. Otherwise, the hypothesis that the influx of Na^+ is the primary event cannot survive because Lederer *et al.* calculated that, without such restricted space, the rise in $[Ca^{2+}]_i$ would be an insignificant 25 μM.

Moreover, Lederer *et al.* point out that this restricted space must be accessible not only to Na^+ channels and the Na^+-Ca^{2+} exchanger, but to both the SR and to L-type Ca^{2+} channels that are believed to contribute to Ca^{2+}-induced Ca^{2+} release from the SR. Indeed, the entire cytoplasm must be accessible to the Na^+-Ca^{2+} exchanger, since J. H. B. Bridge *et al.* (*5*) show that all the nifedipine-sensitive inward calcium flux associated with contractures of cardiac myocytes is extruded by the exchanger.

We agree with Lederer *et al.* that such a space is poorly conceptualized. Its special properties are hard to reconcile with the known cellular architecture of heart cells, so much so that we question its necessity. In the squid giant axon it has been shown (*6*) that there are only two plasmalemmal pathways that lead to an increase in $[Ca^{2+}]_i$; one is by Na^+-Ca^{2+} exchange and the other is through TTX-sensitive Na^+ channels—the latter possibility not considered by Leblanc and Hume or by Lederer *et al.* We suggest that, unless proved otherwise, a Ca^{2+} flux through TTX-sensitive Na^+ channels is a more likely explanation of the findings of Leblanc and Hume than a "fuzzy space" for the accumulation of Na^+, as discussed by Lederer *et al.*

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Response: In our report (*1*) we hypothesized that the influx of Ca^{2+} mediated by the Na^+-Ca^{2+} exchange in response to Na^+ influx through tetrodotoxin (TTX)-sensitive Na^+ channels induces Ca^{2+} release from the sarcoplasmic reticulum (SR) in guinea pig heart myocytes. Johnson and Lemieux suggest that Ca^{2+} influx through voltage-dependent TTX-sensitive Na^+ channels provides the Ca^{2+} trigger for release of SR Ca^{2+}, thereby minimizing the role of Na^+-Ca^{2+} exchange. We initially considered this alternative explanation, but believe such a possibility to be unlikely.

In squid axon there can be significant Ca^{2+} entry through TTX-sensitive Na^+ channels (*2*), however, most experiments were carried out in high extracellular Ca^{2+} ($[Ca^{2+}]_o$) solutions (about 100 mM). These experiments do not, therefore, reveal the extent of Ca^{2+} influx through Na^+ channels that might be expected in our experiments with solutions containing physiological $[Ca^{2+}]_o$ (2.5 mM). More recent studies of the effects of $[Ca^{2+}]_o$ on Na^+ channel permeability in nerve (*3*) and cardiac preparations (*4*) conclude that there is little if any divalent cation permeability and that divalent cations instead block Na^+ channels, which results in a nonlinearity of the instantaneous Na^+ current–voltage relationship.

The hypothesis that Na^+-Ca^{2+} exchange mediates Na^+ current–induced Ca^{2+} release from cardiac SR is supported by experi-