a change in the hydropathy profile[14] of the receptor protein. The defect was not found in the gene of the patient's brother, and both the normal and the mutant allele were amplified from the genomic DNA of the mother.

Patients with CNDI respond to adrenaline and parathyroid hormone (but not to arginine vasopressin or its analogues) with an increase in plasma cyclic AMP concentration and urinary cAMP excretion, respectively[15,16]. Thus, genetic defects in ubiquitous signal transduction components (the G_s/adenylyl cyclase system) as they occur in pseudohypoparathyroidism type Ia[17], are not likely. On the basis of our findings and the observation that CNDI patients generally lack both V2 receptor-mediated renal and extrarenal responses to arginine vasopressin[2], we predict that the disorder will frequently be ascribable to a defect of the V2 receptor gene. □

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APC mutations occur early during colorectal tumorigenesis

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HUMAN tumorigenesis is associated with the accumulation of mutations both in oncogenes and in tumour suppressor genes[1–3]. But in no common adult cancer have the mutations that are critical in the early stages of the tumorigenic process been defined. We have attempted to determine if mutations of the *APC* gene play such a role in human colorectal tumours, which evolve from small benign tumours (adenomas) to larger malignant tumours (carcinomas) over the course of several decades. Here we report that sequence analysis of 41 colorectal tumours revealed that the majority of colorectal carcinomas (60%) and adenomas (63%) contained a mutated *APC* gene. Furthermore, the *APC* gene met two criteria of importance for tumour initiation. First, mutations of this gene were found in the earliest tumours that could be analysed, including adenomas as small as 0.5 cm in diameter. Second, the frequency of such mutations remained constant as tumours progressed from benign to malignant stages. These data provide strong evidence that mutations of the *APC* gene play a major role in the early development of colorectal neoplasms.

The candidate tumour suppressor gene, *APC*[4,5], is abnormal in the germline of patients with familial adenomatous polyposis (FAP), an inherited disease predisposing to colorectal neoplasms[6–8]. But most colorectal neoplasms occur in patients without FAP. Until now, the role of the *APC* protein (APC) in these more common neoplasms has been largely conjectural because only a few *APC* mutations, occurring in advanced colorectal carcinomas, have been described[6]. To determine the role of APC in the development of sporadic colorectal tumours, we examined the *APC* gene for mutations in 41 sporadic

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TABLE 1 Somatic *APC* mutations

Sample*	Size or stage†	Alleles‡	Nucleotide change§	Coding change‖	
Ca-1	B	2	CGA→TGA	Arg→Stop	(Codon 564)
Ca-2	C	2	taatttttag/GGT→tagtttttag/GGT	Splice acceptor	(Exon 8)
			AAA→AA	Deletion	(Codon 2,455)
Ca-4	A	2	CGA→TGA	Arg→Stop	(Codon 876)
			CCT→CC	Deletion	(Codon 1,443)
Ca-6	B	2	AAA→AA	Deletion	(Codon 758)
Ca-8	B	2	TTT→TT	Deletion	(Codon 801)
Ca-10	B	2	AAT→AT	Deletion	(Codon 1,455)
			AAG→G	Deletion	(Codon 1,462)
Ca-12	B	2	CGA→TGA	Arg→Stop	(Codon 232)
			CGA→TGA	Arg→Stop	(Codon 564)
Ca-14	B	2	CGA→TGA	Arg→Stop	(Codon 1,450)
			AAG→G	Deletion	(Codon 1,462)
Ca-16	D	2	AAT→AT	Deletion	(Codon 869)
Ca-18	A	2	TTT→T	Deletion	(Codon 773)
			ATA→AATA	Insertion	(Codon 1,307)
Ca-19	C	1	17 bp deletion (nt 4060–4077)	Deletion	(Codon 1,354)
Ca-20	C	2	GTT→TT	Deletion	(Codon 1,405)
Ca-21	B	2	AAA→TAA	Cys→Stop	(Codon 670)
Ca-22	B	2	TCT→TAT	Ser→Tyr	(Codon 906)
			CGA→TGA	Arg→Stop	(Codon 1,450)
Ca-25	C	1	TCA→TAA	Ser→Stop	(Codon 1,346)
Ad-1	1.1 cm	2	CGA→TGA	Arg→Stop	(Codon 876)
			ACT→AACT	Insertion	(Codon 1,556)
Ad-2	0.8 cm	2	GAA→AA	Deletion	(Codon 403)
Ad-3	0.7 cm	1	AGT→AGAGAGT	Insertion	(Codon 1,465)
Ad-4	1.2 cm	2	tag/GTC→taa/GTC	Splice acceptor	(Exon 4)
			CGA→TGA	Arg→Stop	(Codon 405)
			ACT→AACT	Insertion	(Codon 1,556)
Ad-7	2.5 cm	2	CAG→TAG	Gln→Stop	(Codon 1,367)
Ad-9	0.5 cm	2	CCA→ACCA	Insertion	(Codon 1,941)
Ad-10	1.2 cm	2	CCT→CCCT	Insertion	(Codon 1,373)
Ad-11	0.7 cm	1	TCA→TAA	Ser→Stop	(Codon 1,281)
Ad-12	0.5 cm	2	CGA→TGA	Arg→Stop	(Codon 1,450)
Ad-15	3.5 cm	1	GAA→TAA	Glu→Stop	(Codon 1,408)

* Ad indicates an adenoma and Ca indicates a carcinoma.

† For carcinomas, the modified Dukes' stage[17] is indicated. For adenomas, the largest diameter is given.

‡ Allelic statuses were determined using two dinucleotide repeat polymorphisms located on 5q[18,19], a deletion/insertion polymorphism 3′ to exon 10 of MCC[20,21], and the APC sequence polymorphisms detailed in Table 2.

§ Underlined nucleotides were mutant. Lower case letters represent introns, upper case letters represent exons.

‖ The first affected codon or exon is listed in parentheses.

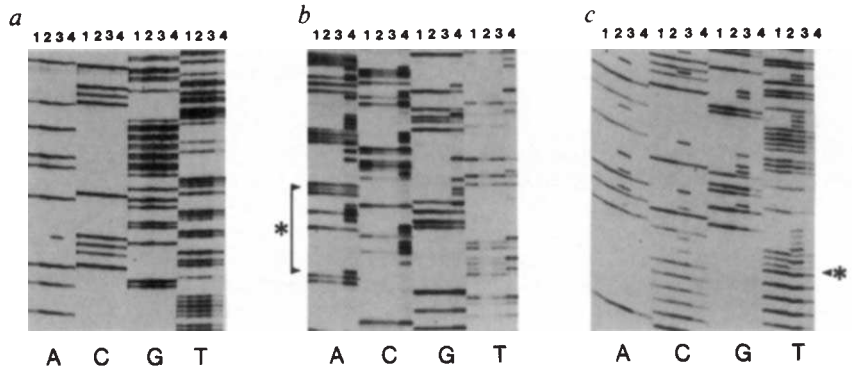

FIG. 1 Screening for *APC* mutations in sporadic colorectal tumours by DNA sequencing. Three representative examples of mutations are illustrated. Autoradiographs of sequencing gels from four different tumours (labelled 1 to 4) are shown in each panel. Sequencing reactions were grouped by nucleotide (A, C, G and T) to aid identification of changes. Carcinoma Ca-22 (lane 3 in *a*) contained a C→T transition at the first base of codon 1,450 creating an 'A' (marked with an asterisk) in the antisense sequence displayed. Carcinoma Ca-19 (lane 4 in *b*) contained a 17 bp deletion affecting codons 1,354 to 1,359 as indicated by an asterisk and bracket on the sense sequence displayed. Adenoma Ad-3 (lane 3 in *c*) contained a 4 bp insertion beginning at codon 1,465 creating a frameshift beginning at the base marked with an asterisk in the antisense sequence shown.

METHODS. DNA was prepared from tumours enriched for neoplastic cells by cryostat sectioning as previously described[11]. Normal DNA was prepared from adjacent nonaffected colonic mucosa. Exons 1 through 14 and surrounding splice sites were individually amplified using PCR and subcloned into a T-tailed pBluescript as previously described[6]. For exon 15, three separate but overlapping fragments, each ~2.3 kb, were amplified with paired primers engineered to contain either an *Eco*RI or *Bam*HI sites at their ends to aid directional subcloning into a *Bam*HI- and *Eco*RI-digested pBluescript vector. Pools of at least 50 clones for each PCR product were sequenced with internal primers as previously described[22]. The 34 amplification primers and 49 sequencing primers were designed from previously reported sequence data[4,7,8] and their sequences are available from the authors on request. Each mutation was confirmed by a second independent PCR amplification and sequence analysis.

colorectal tumours of various size, stage and degree of malignancy. These tumours included 25 carcinomas ranging from localized disease to fully metastatic disease and 16 adenomas ranging from 0.5 cm to 4.5 cm (average 1.6 cm) in diameter. Although rapid screening techniques are often used to search for mutations in genes as large as *APC*, we chose to sequence the entire coding region of the gene in all 41 tumours. Although more difficult, the sequencing approach provides maximum sensitivity for detecting coding and splice-site mutations. For this purpose, individual exons and their surrounding splice sites were amplified from tumour DNA using the polymerase chain reaction (PCR). The amplified DNA was then cloned and sequenced using internal sequencing primers (see legend to Fig. 1). In each case wherein a variant was identified, an independent amplification was used to confirm its validity, and its definition as a somatic mutation was accomplished by comparing the sequence to that found in normal tissues of the same patient.

Fifteen of the 25 (60%) colorectal carcinomas and 10 of 16 (63%) adenomas examined contained at least one somatic mutation of the *APC* gene (Table 1, examples in Fig. 1). Most of the mutations resulted in a truncated APC protein. There was similarity in the character and distribution of mutations in both the adenomas and carcinomas (Table 1). Of the 35 mutations identified, six were insertions ranging from 1 to 4 base pairs (bp) and 12 were deletions ranging from 1 to 17 bp. All of the insertions and deletions produced a frameshift that would truncate the protein. Similarly, 14 of the point mutations found were nonsense mutations whereas only one was a missense mutation. The two remaining somatic mutations altered splice sites and would be expected to result in an *APC* transcript containing a frameshift. One abolished the splice acceptor site for exon 4 (Ad-4, Table 1) and the other created a cryptic splice acceptor sequence 7 bp upstream of exon 8 (Ca-2, Table 1). Of the 17 point mutations, 15(88%) were at G·C bp and 9 of these were at a CpG dinucleotide. This same tendency for mutations to occur at G·C bp and particularly at CpG dinucleotides was also observed in the p53 gene from colorectal tumours[9], suggesting deamination of 5-methylcytosine as a possible mechanism[10].

The majority (90%) of the mutations identified were distributed over the first 55% of the coding region, with half of the mutations occurring in a 722 bp region spanning codons 1281 to 1554 (Fig. 2). These somatic mutations in sporadic colorectal tumours are very similar to those found in the germ line of FAP patients in terms of both type (tuncations) and distribution along the gene[8].

Many of the tumours had alterations of both alleles of their *APC* gene. In five tumours, one copy of the *APC* gene was inactivated by allelic loss and the remaining copy contained an intragenic mutation (Ca-19, Ca-25, Ad-3, Ad-11, Ad-15, Table 1). An additional nine tumours contained two intragenic mutations, presumably one on each allele (Table 1). This presumption was testable in three of these nine cases because the mutations were close enough to be included in one PCR product (Ca-10, Ca-14, Ca-18, Table 1). In all three cases, sequence analysis of individual PCR clones indicated that the mutations were on separate alleles. One tumour had three somatic mutations (Ad-4, Table 1). Histopathology of this tumour revealed two morphologically distinct areas of adenomatous growth suggesting that two independent areas of clonal expansion were present in the sample analysed. In total, 14 of 41 tumours (31% of adenomas, 36% of carcinomas) were predicted to have no normally functioning APC protein. This suggests that there is a growth advantage afforded by mutating both copies of the *APC* gene but that two mutations may not be required for tumorigenesis.

The fact that colorectal adenomas (63%) were just as likely as colorectal carcinomas (60%) to acquire an *APC* mutation suggests that *APC* mutations occur as an early event during colorectal tumorigenesis. Indeed, *APC* mutations were detected in the smallest adenomas that were examined, including five tumours less than 1 cm in diameter (Table 1). We have examined these same five tumours for mutations in the three *ras* genes (K-*ras*, N-*ras*, and H-*ras*), which were heretofore the only genes known to be specifically mutated during the early stages of colorectal tumorigenesis[11]. Only one of the five tumours with *APC* gene mutations contained a *ras* gene mutation, consistent with the idea that *APC* alterations precede those of *ras*.

FIG. 2 Distribution of *APC* mutations. This diagram illustrates the position of all 35 somatic mutations relative to the APC protein and exon structure. The boxes correspond to exons (1, 3, 5, 7, 9, 11, 14 and 15 are numbered for reference) and the numbers below represent amino-acid residues. Truncations arose from nonsense mutations, insertions or deletions as detailed in Table 1.

TABLE 2 Germ line *APC* variants

Frequency*	Nucleotide change†	Coding change‡	
57%/43%	TAC/TAT	Silent	(Codon 486)§‖
62%/38%	GCA/GCG	Silent	(Codon 545)§
63%/37%	ACA/ACG	Silent	(codon 1,493)§
63%/37%	TCG/TCT	Silent	(Codon 1,756)‖
63%/37%	CCA/CCG	Silent	(Codon 1,960)
66%/34%	GGA/GGG	Silent	(Codon 1,678)§‖
96%/4%	GGA/GGG	Silent	(Codon 2,568)
90%/10%	GAC/GTC	Asp/Val	(Codon 1,822)
98%/2%	CCCA/TCA	Pro/Ser	(Codon 870)
99%/1%	GGA/GTA	Gly/Val	(Codon 84)

* Determined by sequence analysis of the 90 alleles in 45 individuals.
† Underlined nucleotides were variant.
‡ The codon affected is listed in parentheses.
§ Reported previously in ref. 8.
‖ Reported previously in ref. 7.

During the course of these studies, several germline variants were identified, most of which were common silent polymorphisms (Table 2). But three variants of *APC* were observed that resulted in amino-acid changes. One variant produced an aspartate to valine change at codon 1,822 and was found in nine of the patients examined. A second variant was a proline to serine change at codon 870, found in two patients. The third was a glycine to valine change at codon 84, found in a single patient. Analysis of the allele frequencies of these three variants using allele specific hybridization in 100 healthy individuals revealed no significant difference between the normal population and the patient population examined initially (data not shown). This is interesting in light of the suggestion that there is a common inherited predisposition to colorectal neoplasia affecting 19% of the population[12]. At this point, it is not possible to determine whether these amino-acid changes result in an inherited colorectal tumour risk or are simply inconsequential variants.

Our studies probably underestimate the true prevalence of APC abnormalities in sporadic colorectal tumours for two reasons. First, the current analysis did not include the promoter region or intronic portions of the *APC* gene. Mutations localized in these regions could also result in loss of APC activity. Second, this analysis only considered intragenic mutations of the *APC* gene, not large deletions that could also inactivate the *APC* gene. Indeed, allelic loss analysis revealed three additional tumours that had lost one copy of the *APC* gene but did not contain a detectable *APC* mutation; bringing the total to 28 (68%) tumours that had either mutated or lost at least one copy of the *APC* gene.

The high prevalence of *APC* gene mutations in the colorectal tumours examined provides strong *in vivo* evidence that APC alterations play a major role in the development of most common colorectal tumours. Furthermore, the data show that *APC* mutations are a very early event in colorectal tumorigenesis, if not the initiating event. The similar frequency of *APC* mutations as tumours progress from benign to malignant stages stands in marked contrast to other changes, such as inactivation of the p53 tumour suppressor gene, which increases in frequency as tumours advance[13]. These data also indicate that the small adenomas occurring in FAP patients and those that occur sporadically have a similar molecular defect: a mutation in *APC*, inherited in the former case and somatically acquired in the latter. This similarity has important practical as well as biological implications. First, nonsteroidal anti-inflammatory drugs reverse the growth of colorectal adenomas in FAP patients[14]. Such drugs might also find use in chemotherapy of sporadic tumours if the underlying pathogenesis is the same. Second, an animal model for FAP has been described in the form of a mouse lineage which contains a germ line mutation of the *APC* gene[15]. Because of this shared genetic defect, tumours in these mice may prove to be a good model for sporadic colorectal tumours as well as for FAP. Finally, a potential screening test for colorectal tumours based on the detection of mutated genes from tumour cells shed into the stool was recently described[16]. Such a test might optimally be done using probes for genes that are mutated frequently and early in the neoplastic process, long before malignancy. The *APC* gene might therefore provide special opportunities in this regard. □

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Evidence that Hensen's node is a site of retinoic acid synthesis

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HENSEN'S node of amniotes, like the Spemann organizer of amphibians, can induce a second body axis when grafted into a host embryo[1]. The avian node, as well as several midline structures originating from it (notochord, floor plate), can also induce digit pattern duplications when grafted into the chick wing bud[2,3]. We report here that the equivalent of Hensen's node from mouse is an effective inducer of digits in the chick wing bud. Tissues anterior and posterior to the node also evoke pattern duplications, but with a significantly lower efficiency. The finding that the murine node operates in an avian wing bud suggests that the same inducing agent(s) function in both primary and secondary embryonic fields and have been conserved during vertebrate evolution. Digit pattern duplications are also evoked by local administration of all-*trans*-retinoic acid[4,5]. This similarity raises the possibility that Hensen's node is a source of retinoic acid. The mouse node is capable of synthesizing retinoic acid from its biosynthetic precursor all-*trans*-retinol at a substantially higher rate than either anterior or posterior tissues.

To map the digit-inducing capacity of mouse embryonic tissues, intact embryos or dissected fragments ranging from the pre-streak, egg cylinder stage (6.5 days) to embryos with 2-3 somites (7.75 days) were implanted into chick wing buds. Egg cylinder stage embryos failed to induce additional digits (data

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