



Constitutive Transcriptional Activation by a beta-catenin-Tcf complex in APC-/- Colon Carcinoma

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22. Congenic wild-type (WT) (Y762), *bck1Δ:TRP1* (Y782), *sit2Δ:LEU2* (Y783), and *swi4Δ:HIS3* (YK74) strains were prepared (11, 16). High-copy plasmids containing the yeast 2 μ origin of replication and either *SLT2* (16), *PCL1* (11), *PCL2* (pBA623; Hind III-Sal I fragment from a genomic library plasmid cloned into pRS426), *PHO85* (BK55; YE24 genomic library plasmid), *SWI4* (B327; 8-kb Sph I fragment including *SWI4* subcloned into YE352), or *MBP1* [BK72; polymerase chain reaction product including *MBP1* and flanking sequence (shown to be functional) cloned into YE24] were transformed into *sit2Δ* and *bck1Δ* strains. These plasmids are expected to be present at ~20 copies per cell (33). Transformants were streaked on plates containing synthetic media lacking uracil (34) and incubated for 3 days at 37°C.

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24. *sit2Δ* cells containing either YE24, or *SWI4*, *PCL1*, or *PCL2* high-copy plasmids (Fig. 1) were grown to late-log phase in 5-ml cultures of synthetic medium lacking uracil (with or without 1 M sorbitol); cells were diluted to an absorbance at 600 nm of 0.1 and grown for 3 hours in fresh synthetic complete medium. Cells were then centrifuged, resuspended, and incubated for 2 hours in 5 ml of rich medium with or without 1 M sorbitol. α -Factor (Sigma) was then added (5 μ g/ml), and after 1 hour an equivalent dose of pheromone was added to the culture. The incubation was continued for 1 hour, and cells were fixed with 3.7% formaldehyde and washed three times with phosphate-buffered saline. Cells were observed by differential-interference microscopy, and the level of suppression of *sit2Δ* mutant phenotypes was quantified. Cells were either lysed, budded, or arrested in response to pheromone. Cells arrested in response to mating factor were divided into two classes on the basis of projection length: either \geq or $<$ one-third the diameter of the cell at the widest point. "Other" category includes cells with more than one projection or cells that have undergone polarized growth events that could not clearly be classified as buds or mating projections.

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27. Affinity-purified anti-*Swi6* was used to immunoprecipitate *Swi6p* from 1 mg of total protein isolated from cells containing high-copy *SLT2:HA*; the cells had been incubated at 39°C for 1 hour. Immunoprecipitates were resuspended in 45 μ l of NEB (New England Biolabs) Buffer 3 with 1% SDS. After 5 min of incubation at 37°C, the supernatant was removed and diluted to 0.1% SDS in NEB Buffer 3 with either 30 U of calf intestinal alkaline phosphatase (CIP) or 30 U of CIP together with 4 mM sodium molybdate (CIP + inhib.). Phosphatase reactions were incubated for 30 min at 37°C, and then *Swi6p* was reimmunoprecipitated from the phosphatase reaction.

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29. Recombinant baculoviruses containing *SWI4* and *SWI6* were isolated from SF9 insect cells and coinjected into H9c2 cells, as recommended (Invitrogen). Two days after infection, cells were harvested, washed, and lysed in buffer D [50 mM tris-HCl (pH 8.0), 20% glycerol, 50 mM NaCl, 0.5 mM EDTA, 10 mM β -mercaptoethanol], yielding ~2.5 mg of total protein per milliliter. To separate SBF from insect cell kinase activity, we precipitated *Swi4p* and *Swi6p* by addition of ammonium sulfate to a final concentration of 20%. The sedimented protein was resuspended in buffer D and loaded onto a heparin-agarose column for further purification (Affigel Heparin, Bio-Rad). *Swi4p* and *Swi6p* were eluted in 300 to 500 mM (buffer D plus increasing NaCl) washes and concentrated to 0.1 mg/ml. Five microliters of partially purified protein was used per kinase reaction. *Swi4p* was immunodepleted by addition of 2 μ l of antibodies (~25 mg/ml) to *Swi4p* to 10 μ l of the protein preparation for 5 hours at 4°C followed by addition of 5 μ l of protein A-Sepharose (Pierce) to this mixture. After a 2-hour incubation the supernatant was removed. *Slt2:3HAp* IP and kinase reactions were similar to those described (28), except that for each kinase reaction 250 μ g of total yeast protein was used for the *Slt2:3HAp* IP. In addition, the kinase reaction was incubated 15 min before the addition of [γ -32P]ATP, and the reaction was allowed to proceed for 50 min. Half of each reaction was separated in a 10% polyacrylamide gel and blotted; the filter was used for autoradiography.

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36. Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE; 10% gel), blotted onto Immobilon-P (Millipore), probed with affinity-purified anti-*Swi6p*, and primary antibodies were detected with alkaline phosphatase-conjugated goat anti-rabbit IgG and the bromochloroindolyl phosphate-nitro blue tetrazolium (BCIP-NBT) color substrate reaction (35).

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38. Samples for two-dimensional electrophoresis were suspended in SDS sample buffer, then treated so that the first-dimension sample buffer contained 9.5 M urea, 2.0% Triton X-100, 5% β -mercaptoethanol, and 1.6% Bio-Lyte 5/7 and 0.4% Bio-Lyte 3/10 ampholytes (Bio-Rad). First-dimension isoelectric focusing and second-dimension SDS-PAGE (10% gel) were performed as recommended (Mini-PROTEAN II, Bio-Rad). The SDS-PAGE gel was blotted (36) and exposed for autoradiography (29).

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Constitutive Transcriptional Activation by a β -Catenin-Tcf Complex in APC^{-/-} Colon Carcinoma

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The adenomatous polyposis coli (APC) tumor suppressor protein binds to β -catenin, a protein recently shown to interact with Tcf and Lef transcription factors. The gene encoding hTcf-4, a Tcf family member that is expressed in colonic epithelium, was cloned and characterized. hTcf-4 transactivates transcription only when associated with β -catenin. Nuclei of APC^{-/-} colon carcinoma cells were found to contain a stable β -catenin-hTcf-4 complex that was constitutively active, as measured by transcription of a Tcf reporter gene. Reintroduction of APC removed β -catenin from hTcf-4 and abrogated the transcriptional transactivation. Constitutive transcription of Tcf target genes, caused by loss of APC function, may be a crucial event in the early transformation of colonic epithelium.

The product of the APC tumor suppressor gene has been observed to interact with β -catenin and has thus been proposed to regulate cellular signaling events (1). β -Catenin, originally identified on the basis of its association with cadherin adhesion molecules, is now widely recognized as an essential element of the Wingless-Wnt sig-

naling cascade (2). In the absence of Wnt signals, APC simultaneously interacts with the serine kinase glycogen synthase kinase (GSK)-3 β and with β -catenin. Phosphorylation of APC by GSK-3 β regulates the interaction of APC with β -catenin, which in turn may regulate the signaling function of β -catenin (3). Wnt signaling appears to

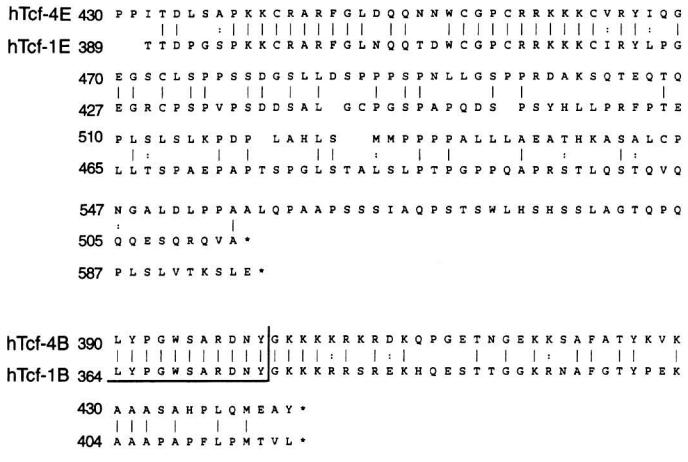
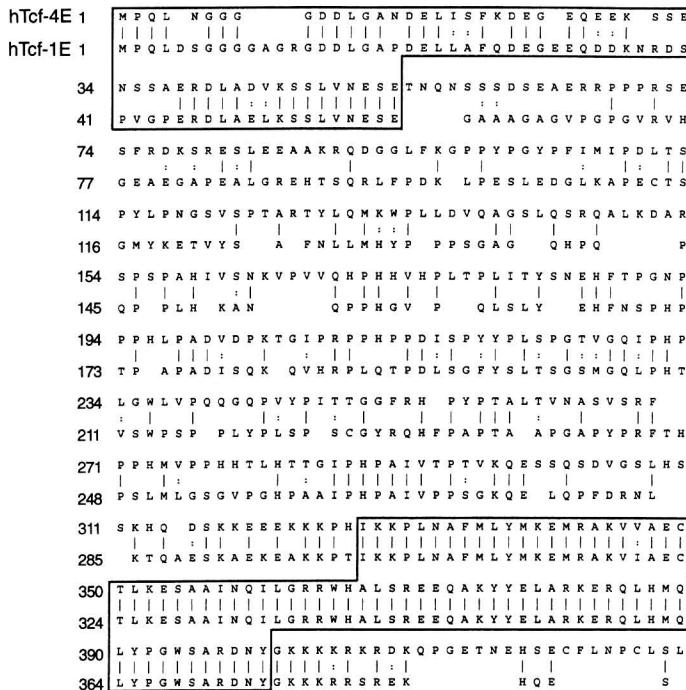


Fig. 1. Sequence comparison of hTcf-4 and hTcf-1. Two alternative splice forms of *hTcf-4* were identified, each encoding a different COOH-terminus. One form (hTcf-4E) was homologous to hTcf-1E (7); the other form (hTcf-4B) was homologous to hTcf-1B. The highly conserved NH₂-terminal interaction domain and the HMG-box DNA-binding region are boxed. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. The nucleotide sequence has been deposited in the European Molecular Biology Laboratory database (accession number Y11306). Symbols: |, sequence identity; :, sequence similarity.

antagonize GSK-3 β activity. Upon Wnt signaling, β -catenin is stabilized and exists primarily as a cytoplasmic monomer (4). Colon carcinoma cells with mutant APC contain large amounts of monomeric, cytoplasmic β -catenin. Reintroduction of wild-type APC removes this cytoplasmic pool and reduces the overall amount of β -catenin (5). Recent evidence indicates that monomeric β -catenin can transduce Wnt signals by associating with T cell factor (Tcf) and lymphoid enhancer factor (Lef) transcription factors (6). We hypothesized that APC may regulate the formation of transcriptionally competent β -catenin-Tcf complexes. If so, loss of APC function would result in uncontrolled transcriptional activation of Tcf target genes, which might contribute to colon tumorigenesis.

There are four known members of the Tcf and Lef family in mammals: the lymphoid-specific factors Tcf-1 and Lef-1 (7, 8) and the less well characterized Tcf-3 and Tcf-4 (9). We performed a qualitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay for expression of the

four Tcf-Lef genes on 43 colon tumor cell lines. Although most colon cell lines expressed more than one of the genes, only hTcf-4 mRNA was expressed in essentially all lines (10).

We then screened a human fetal cDNA library and retrieved clones encoding full-length hTcf-4 (11) (Fig. 1). The predicted sequence of hTcf-4 was most similar to that of hTcf-1. Alternative splicing yielded two COOH-termini that were conserved be-

tween hTcf-1 and hTcf-4. The NH₂-terminus, which mediates binding to β -catenin in hTcf-1, mouse Lef-1, and *Xenopus* TCF-3 (6), was also conserved in hTcf-4. Northern (RNA) blot analysis of selected colon carcinoma cell lines (12) revealed extensive expression of hTcf-4 (Fig. 2A). As evidenced by in situ hybridization (Fig. 2, B and C) (13) and Northern blotting (Fig. 2A), hTcf-4 mRNA was readily detectable in normal colonic epithelium, whereas

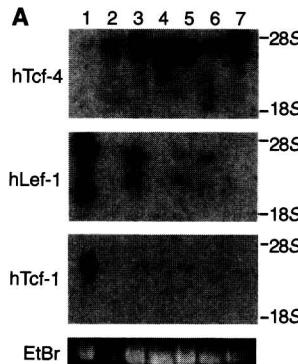


Fig. 2. Analysis of hTcf-4 expression. (A) Northern blot analysis of hTcf-4, hTcf-1, and hLef-1 expression in Jurkat T cells (lane 1), colonic mucosa (lane 2), and colon carcinoma cell lines DLD-1 (lane 3), HCT116 (lane 4), SW480 (lane 5), SW620 (lane 6), and HT29 (lane 7). Lane 2 contains 5 μ g of total RNA; all other lanes contain 15 μ g of total RNA. The positions of 18S and 28S ribosomal RNAs are shown. EtBr, ethidium bromide stain. (B) In situ hybridization of healthy human colon tissue to an hTcf-4 probe. (C) In situ hybridization to a negative control probe (a fragment of the *Escherichia coli* neomycin resistance gene). Magnifications, $\times 166$.

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hTcf-1 and *hLef-1* were not detectable.

To investigate whether *hTcf-4* functionally interacts with β -catenin, we used two sets of reporter constructs in a β -catenin-Tcf reporter gene assay (7, 14). One set contained three copies of the optimal Tcf motif CCTTTGATC, or three copies of the mutant motif CCTTTGGCC, upstream of a minimal *c-Fos* promoter driving luciferase expression (pTOPFLASH and pFOPFLASH, respectively). The second set contained three copies of the optimal motif, or three copies of the mutant motif, upstream of a minimal herpes virus thymidine kinase promoter driving chloramphenicol acetyltransferase (CAT) expression (pTOPCAT and pFOPCAT, respectively). Epitope-tagged *hTcf-4* and a deletion mutant lacking the NH₂-terminal 30 amino acids (Δ NhTcf-4) were cloned into the expression vector pCDNA. Transient transfections were performed in a murine B cell line (IIA1.6) that does not express any of the Tcf genes (6).

The pTOPFLASH reporter was strongly transcribed upon cotransfection with the combination of β -catenin and *hTcf-4* plasmids, but not with the individual plasmids or with the combination of β -catenin and Δ NhTcf-4 plasmids. No enhanced transcription was detected in cells transfected with the negative control pFOPFLASH (Fig. 3A). These results show that interaction of the NH₂-terminus of *hTcf-4* with β -catenin results in transcriptional activation.

In three APC⁺ carcinoma cell lines, SW480, SW620, and DLD-1 (15), the transcriptional activity of the pTOPFLASH reporter was 5 to 20 times that of pFOPFLASH. Cotransfection of SW480 cells with the reporter gene and an APC expression vector abrogated the transcriptional activity in a dose-dependent manner (Fig. 3B). In contrast, APC had no effect on a cotransfected internal control (pCAT-

CONTROL) or on the basal transcription of pFOPFLASH (Fig. 3B). The use of pTOPCAT and pFOPCAT instead of pTOPFLASH and pFOPFLASH led to comparable observations. The constitutive transcriptional activity of *Tcf* reporter genes in APC⁺ colon carcinoma cells was in stark contrast to the inactivity of these genes in noncolon carcinoma cell lines, including IIA1.6 B cells (Fig. 3A); the C57MG breast carcinoma cell line; the Jurkat and BW5147 T cell lines; the Daudi and NS1 B cell lines; the K562 erythromyeloid cell line; the HeLa cervical carcinoma line; the HepG2 hepatoma cell line; 3T3, 3T6, and Rat-1 fibroblasts; and the kidney-derived SV40-transformed COS cell line (7, 16).

To investigate whether a functional β -catenin-*hTcf-4* complex exists constitutively in APC⁺ cells, we used HT29-APC1 colon carcinoma cells (17), in which APC is controlled by a metallothionein promoter. Induction by Zn²⁺ restores wild-type

amounts of APC and leads to apoptosis (17). HT29-Gal cells that carry a Zn²⁺-inducible *LacZ* gene were used as a control. The only Tcf family member expressed in HT29 is *hTcf-4* (Fig. 2A). In nuclear extracts from uninduced HT29-derived transfected cells, we readily detected *hTcf-4* by gel retardation (Fig. 4) (18). An additional band of slightly slower mobility was also observed. The addition of a β -catenin antibody resulted in the specific retardation of the latter band, indicating that it represented a β -catenin-*hTcf-4* complex (Fig. 4) (17). After Zn²⁺ induction for 20 hours, the amount of β -catenin-*hTcf-4* complex was reduced by five-sixths in HT29-APC1 cells, whereas no marked change was observed in HT29-Gal cells (Fig. 4). The overall amount of cellular β -catenin does not change during this induction period in HT29-APC1 cells (17).

On the basis of these data, we propose the following model. In normal colonic epithelium, *hTcf-4* is the only expressed member of the Tcf family. The interaction of β -catenin with *hTcf-4* is regulated by APC. When appropriate extracellular signals are delivered to an epithelial cell, β -catenin accumulates in a form that is not complexed with GSK-3 β -APC and that enables its nuclear transport and association with *hTcf-4*. The high mobility group (HMG) domain of *hTcf-4* binds in a sequence-specific fashion to the regulatory sequences of specific target genes; β -catenin supplies a transactivation domain. Thus, transcriptional activation of target genes occurs only when *hTcf-4* is associated with β -catenin. The *hTcf-4* target genes remain to be identified. However, the link with APC and β -catenin suggests that these genes may participate in the generation and turnover of epithelial cells. Upon the loss of wild-type APC, monomeric β -catenin accumulates in the absence of extracellular

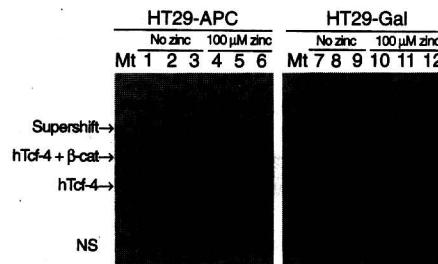


Fig. 4. Constitutive presence of β -catenin-*hTcf-4* complexes in APC⁺ cells. Gel retardation assays were performed on nuclear extracts from the indicated cell lines before and after a 20-hour exposure to Zn²⁺. Samples in lanes 1, 4, 7, and 10 were incubated under standard conditions. Anti- β -catenin (0.25 μ g) was added to the samples in lanes 2, 5, 8, and 11. A control antibody (human CD4, 0.25 μ g) was added to the samples in lanes 3, 6, 9, and 12. NS, nonspecific band also observed with mutant (nonbinding) probe (lane Mt).

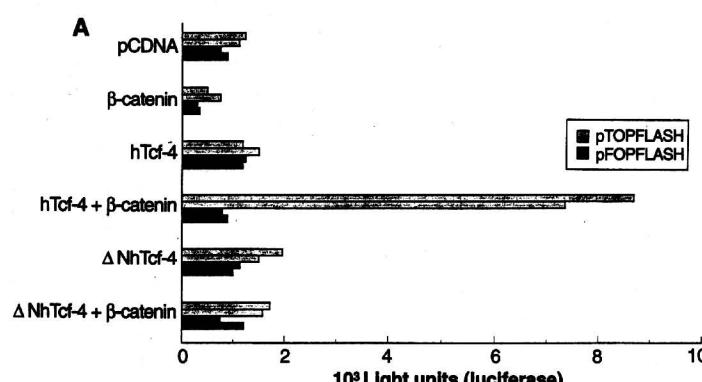
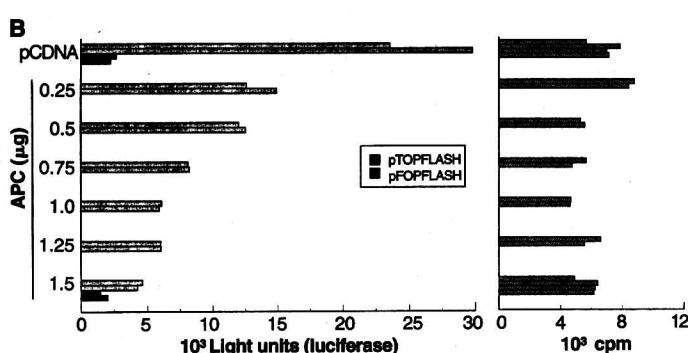


Fig. 3. Transactivation properties of β -catenin-*hTcf-4*. All reporter assays were performed as duplicate transfections. For each condition, both values are shown. (A) Reporter gene assays in IIA1.6 B cells. Cells were transfected by electroporation with 1 μ g of luciferase reporter plasmid, 5 μ g of β -catenin expression plasmid, and 3 μ g of *hTcf-4* expression plasmids. Empty pCDNA was added to



a total of 10 μ g of plasmid DNA. (B) Reporter gene assays in SW480 colon carcinoma cells. Cells were transfected with 0.3 μ g of the indicated luciferase reporter gene, 0.7 μ g of pCATCONTROL as internal control, the indicated amounts of pCMVNeo-APC, and empty pCDNA to a total of 2.5 μ g of plasmid DNA. Control CAT values (pCATCONTROL) are given in the right panel.

stimuli, leading to uncontrolled transcription of the *hTcf-4* target genes. The apparent de novo expression of other members of the Tcf family in some colon carcinoma cell lines might lead to a further deregulation of Tcf target gene expression by the same mechanism. The control of β -catenin-Tcf signaling is likely to be an important part of the gatekeeper function of APC (19), and its disruption may be an early step in malignant transformation.

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11. A genomic fragment encoding the HMG-box region of *hTcf-4* (7) was used to probe a human 12-week fetal cDNA library in Lambda GT-11. Positive clones were subcloned into pBluescriptSK and sequenced.
12. Northern blot hybridizations (7) were performed with full-length *hTcf-1*, *hLef-1*, and *hTcf-4* cDNA. Colon epithelial cells were freshly prepared from a mucosal preparation dissected from a healthy surgical colon sample. The sample was minced and incubated with 1 mM dithiothreitol (DTT) in Hanks' medium to remove mucus. Single-cell suspensions were prepared by incubation at room temperature in 0.75 mM EDTA in Hanks' medium. Epithelial cells were separated from lymphocytes by Percoll gradient centrifugation.
13. *In situ* hybridization of 6 μ m frozen sections of healthy colon biopsy samples was performed as described [E. van Hoffen *et al.*, *Am. J. Pathol.* **149**, 1991 (1996)]. *hTcf-4* cDNA encoding amino acids 200 to 310 was amplified and labeled with Dig-11-dUTP (Boehringer Mannheim) by PCR. After hybridization and washing, the sections were sequentially incubated with mouse antibody to Dig (Boehringer) and a horseradish peroxidase-conjugated rabbit antibody to mouse immunoglobulin (Dako, Glostrup, Denmark). The signal was visualized with diaminobenzidine, which produces a reddish-brown precipitate. Blue counterstaining was performed with hematoxylin.
14. Reporter gene assays were performed as in (7). In brief, 2×10^6 cells were transfected with plasmids by electroporation. After 24 hours, cells were harvested and lysed in 1 mM DTT, 1% Triton X-100, 15% glycerol, 25 mM tris (pH 7.8), and 8 mM MgCl₂. Complementary DNAs encoding Myc-tagged versions of β -catenin and *hTcf-4* were inserted into the mammalian expression vector pCDNA (Invitrogen). Sequences of pTOPCAT, pTOPCAT, pTOPFLASH, and pTOPFLASH are available upon request. pCAT-CONTROL, encoding the CAT enzyme under the control of the SV40 promoter, was purchased from Promega.
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17. P. J. Morin, B. Vogelstein, K. W. Kinzler, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7950 (1996).
18. Gel retardation assays were performed as described (7). Extracts were prepared from intact nuclei that were washed four times to avoid contamination with cytoplasmic β -catenin. As the optimal Tcf-Lo probe, we used a double-stranded 15-nucleotide oligomer CCCTTTGATCTTAC; the control probe was CCCTTTGGCTTAC. All oligonucleotides were from Isogen (Maarssen, Netherlands). The β -catenin antibody was purchased from Transduction Laboratories (Lexington, KY). A typical binding reaction contained 3 μ g of nuclear protein, 0.1 ng of radiolabeled probe, and 100 ng of deoxyinosine-deoxyuridine (dIdC) in 25 μ l of binding buffer (60 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol). Samples were incubated for 20 min at room temperature, antibody was added, and the samples were incubated for a further 20 min.
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Activation of β -Catenin-Tcf Signaling in Colon Cancer by Mutations in β -Catenin or APC

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Inactivation of the adenomatous polyposis coli (APC) tumor suppressor gene initiates colorectal neoplasia. One of the biochemical activities associated with the APC protein is down-regulation of transcriptional activation mediated by β -catenin and T cell transcription factor 4 (Tcf-4). The protein products of mutant APC genes present in colorectal tumors were found to be defective in this activity. Furthermore, colorectal tumors with intact APC genes were found to contain activating mutations of β -catenin that altered functionally significant phosphorylation sites. These results indicate that regulation of β -catenin is critical to APC's tumor suppressive effect and that this regulation can be circumvented by mutations in either APC or β -catenin.

Mutations of the APC gene are the most common disease-causing genetic events in humans; about 50% of the population will develop colorectal polyps initiated by such mutations during a normal life-span (1). Individuals who inherit APC mutations develop thousands of colorectal tumors, consistent with the tumor suppressor or "gatekeeping" role of APC protein in colorectal tumorigenesis (2, 3). APC homodimerizes through its NH₂-terminus (4) and interacts with at least six other proteins: β -catenin (5), γ -catenin (plakoglobin) (6), tubulin (7), EB1 (8), hDLG, a homolog of the *Drosophila* Discs Large tumor suppressor protein (9), and glycogen synthase kinase-3 β (GSK-3 β) (10), a mammalian homolog of ZW3 kinase. Whether any of these interacting proteins communicate APC growth-controlling signals is unknown. Here, we used a genetic

approach to investigate the role of β -catenin in APC's tumor suppressor function.

Although β -catenin was originally discovered as a cadherin-binding protein, it has recently been shown to function as a transcriptional activator when complexed with members of the Tcf family of DNA binding proteins (11). One family member, hTcf-4, is expressed in normal and neoplastic colorectal epithelium, and wild-type (WT) APC can suppress signaling by the β -catenin-Tcf complex (12). If this inhibitory activity is critical for APC's tumor suppressor function, then mutant APC proteins should be defective in this activity.

To evaluate this hypothesis, we tested four APC mutants (Fig. 1A) for their ability to inhibit β -catenin-Tcf-regulated transcription (CRT) in transfection assays. The first mutant, APC331 Δ , represents a type of mutation commonly found in the germ line of familial adenomatous polyposis patients as well as in sporadic tumors (2). The APC331 Δ protein is truncated at codon 331, NH₂-terminal to the three 15-amino acid (aa) β -catenin-binding repeats between codons 1020 and 1169. The second mutant, APC1309 Δ , is the most common germline APC mutation (2), a 5-base pair (bp) deletion that produces a frameshift at

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