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39. We thank C. Mann for strains and plasmids, Y. Ho for providing antibodies and extracts, and Y. Barral, C. Costigan, and C. Crews for critical comments on the manuscript. Supported by grants from the National Institutes of Health (GM36494) (M.S.), the Medical Research Council of Canada (B.A.), a Howard Hughes Medical Institute predoctoral fellowship (K.M.), and a Connaught predoctoral scholarship (K.B.).

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

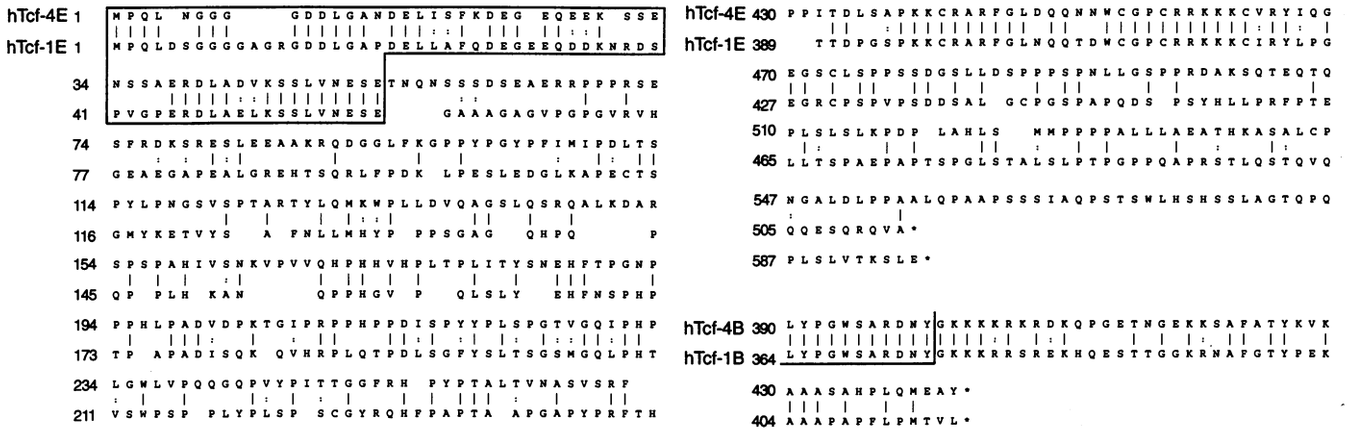

Fig. 1. Sequence comparison of hTcf-4 and hTcf-1. Two alternative splice forms of *hTcf-4* were identified, each encoding a different COOH-terminus. One form (hTcf-4E) was homologous to hTcf-1E (*7*); the other form (hTcf-4B) was homologous to hTcf-1B. The highly conserved NH_2-terminal interaction domain 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-3β 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 between hTcf-1 and hTcf-4. The NH_2-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

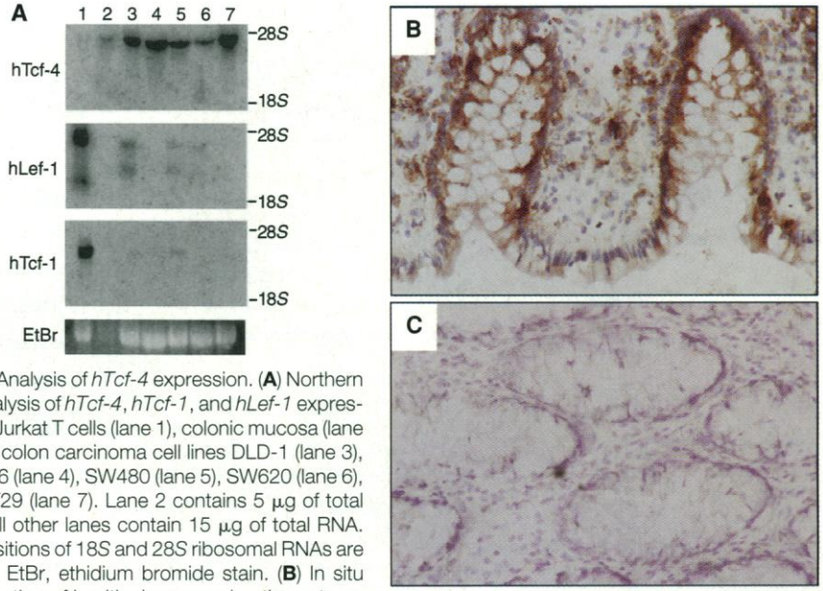

Fig. 2. Analysis of *hTcf-4* expression. (**A**) Northern blot analysis of *hTcf-4*, *hTcf-1*, and *hLef-1* expression in Jurkat T cells (lane 1), colonic mucosa (lane 2), and colon carcinoma cell lines DLD-1 (lane 3), HCT116 (lane 4), SW480 (lane 5), SW620 (lane 6), and HT29 (lane 7). Lane 2 contains 5 μg of total RNA; all other lanes contain 15 μg of total RNA. The positions of 18*S* and 28*S* 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, ×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 NH_2-terminal 30 amino acids (ΔNhTcf-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 ΔNhTcf-4 plasmids. No enhanced transcription was detected in cells transfected with the negative control pFOPFLASH (Fig. 3A). These results show that interaction of the NH_2-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 (pCATCONTROL) 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 noncolonic 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 Zn^{2+} restores wild-type amounts of APC and leads to apoptosis (*17*). HT29-Gal cells that carry a Zn^{2+}-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 transfectants, 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 Zn^{2+} 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*).

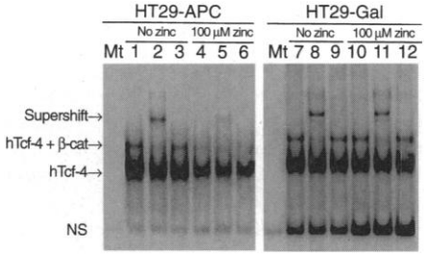

Fig. 4. Constitutive presence of β-catenin–hTcf-4 complexes in $APC^{-/-}$ cells. Gel retardation assays were performed on nuclear extracts from the indicated cell lines before and after a 20-hour exposure to Zn^{2+}. Samples in lanes 1, 4, 7, and 10 were incubated under standard conditions. Anti–β-catenin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

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-3β–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

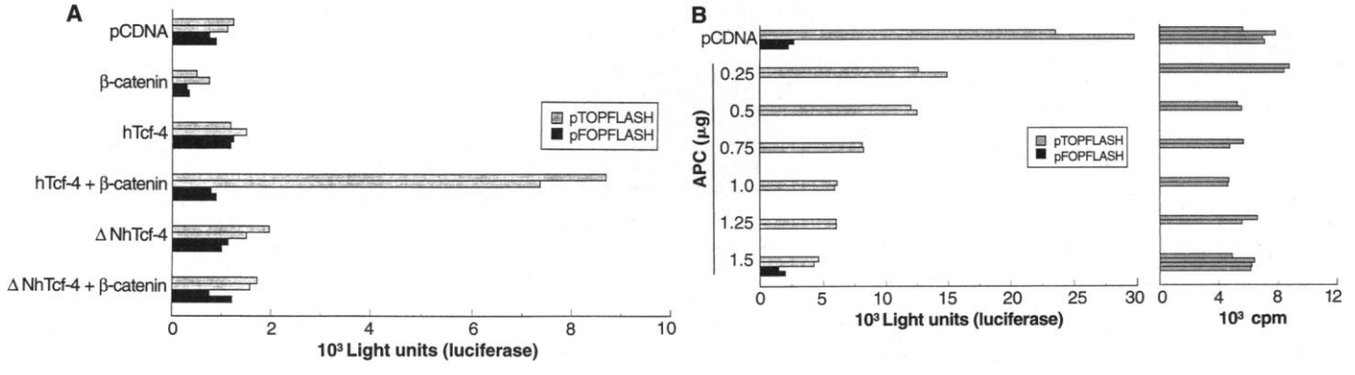

Fig. 3. Transactivational properties of β-catenin–hTcf-4. All reporter assays were performed as duplicate transfections. For each condition, both values are shown. (**A**) Reporter gene assays in IIA1.6 B cells. Cells were transfected by electroporation with 1 μg of luciferase reporter plasmid, 5 μg of β-catenin expression plasmid, and 3 μg of hTcf-4 expression plasmids. Empty pCDNA was added to a total of 10 μg of plasmid DNA. (**B**) Reporter gene assays in SW480 colon carcinoma cells. Cells were transfected with 0.3 μg of the indicated luciferase reporter gene, 0.7 μg of pCATCONTROL as internal control, the indicated amounts of pCMVNeo-APC, and empty pCDNA to a total of 2.5 μg 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 μm 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 $MgCl_2$. Complementary DNAs encoding Myc-tagged versions of β-catenin and hTcf-4 were inserted into the mammalian expression vector pCDNA (Invitrogen). Sequences of pTOPCAT, pFOPCAT, pTOPFLASH, and pFOPFLASH 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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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-Lef probe, we used a double-stranded 15-nucleotide oligomer CCCTTTGATCTTACC; the control probe was CCCTTTGGCCTTACC. All oligonucleotides were from Isogen (Maarssen, Netherlands). The β-catenin antibody was purchased from Transduction Laboratories (Lexington, KY). A typical binding reaction contained 3 μg of nuclear protein, 0.1 ng of radiolabeled probe, and 100 ng of deoxyinosine-deoxycytidine (dIdC) in 25 μl 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 NH_2-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–3β (GSK-3β) (*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, APC331Δ, represents a type of mutation commonly found in the germ line of familial adenomatous polyposis patients as well as in sporadic tumors (*2*). The APC331Δ protein is truncated at codon 331, NH_2-terminal to the three 15–amino acid (aa) β-catenin–binding repeats between codons 1020 and 1169. The second mutant, APC1309Δ, is the most common germline *APC* mutation (*2*), a 5–base pair (bp) deletion that produces a frameshift at

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