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► **To cite this version:**

L. Levy, C. Neuveut, C. A. Renard, P. Charneau, S. Branchereau, et al.. Transcriptional activation of interleukin-8 by beta-catenin-Tcf4. *Journal of Biological Chemistry*, 2002, 277 (44), pp.42386–42393. <10.1074/jbc.M207418200>. <hal-02197533>

HAL Id: hal-02197533

<https://hal.science/hal-02197533v1>

Submitted on 27 May 2021

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Transcriptional Activation of Interleukin-8 by β -Catenin-Tcf4*

Received for publication, July 24, 2002, and in revised form, August 23, 2002
Published, JBC Papers in Press, August 27, 2002, DOI 10.1074/jbc.M207418200

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Nuclear translocation of β -catenin and its association with Tcf/Lef factors are key steps in transduction of the Wnt signal, which is aberrantly activated in a variety of human cancers. In a search for new β -catenin-Tcf target genes, we analyzed β -catenin-induced alterations of gene expression in primary human hepatocytes, after transduction of either dominant stable β -catenin or its truncated, transactivation-deficient counterpart by means of a lentiviral vector. cDNA microarray analysis revealed a limited set of up-regulated genes, including known Wnt targets such as matrilysin and keratin-1. In this screen, we identified the CXC chemokine interleukin 8 (IL-8) as a direct target of β -catenin-Tcf4. IL-8 is constitutively expressed in various cancers, and it has been implicated in tumor progression through its mitogenic, motogenic, and angiogenic activities. The IL-8 promoter contains a unique consensus Tcf/Lef site that is critical for IL-8 activation by β -catenin. We show here that the p300 coactivator was required for efficient transactivation of β -catenin on this promoter. Ectopic expression of β -catenin in hepatoma cells promoted IL-8 secretion, which stimulated endothelial cell migration. These data define IL-8 as a Wnt target and suggest that IL-8 induction by β -catenin might be implicated in developmental and tumorigenic processes.

The canonical Wnt/Wingless signaling pathway plays a pivotal role in regulating growth and cell fate in early and late stages of development (1, 2). These effects are achieved through the stabilization of β -catenin and its translocation to the nucleus as a coactivator for high mobility group-box proteins of the Tcf/Lef family (3, 4). In the absence of Wnt, a multiprotein complex including the protein kinase GSK3 β , adenomatous polyposis coli, and Axin induces phosphorylation of β -catenin at N-terminal serine and threonine residues, and phosphorylated β -catenin is directed toward proteasome-mediated degradation (5, 6). Activation of Wnt abrogates the degradation pathway, leading to elevated levels of transcriptionally active β -catenin (7). Nuclear accumulation of β -catenin induces a transcriptional switch, in which Tcf-bound repressors (CtBP, TLE/Groucho, HDAC) are displaced by β -catenin and its associated coactivators

cAMP-response element-binding protein-binding protein/p300, Brg-1, TIP49/Pontin-52, and Bcl9-pygopus (8–12). The selective activation of distinct Wnt target genes in proper context is strictly controlled by the interplay of positive and negative regulatory signals on Wnt-responsive promoters (13, 14).

Aberrant activation of Wnt signaling is also implicated as a major step in the development of various forms of human cancer (15). De-regulation of β -catenin in cancer results mainly from genetic defects in the N-terminal region of the β -catenin gene itself or in adenomatous polyposis coli or Axin genes. The role of β -catenin is predominant at early steps of colon carcinogenesis, in which truncating mutations of adenomatous polyposis coli leading to elevated levels of β -catenin account for about 80% of cases, whereas stable dominant β -catenin mutants are present in one-half of the remaining cases (16). Activation of Wnt signaling in hepatocellular carcinoma is mainly associated with missense mutations of the β -catenin gene in about 20% of cases and loss-of-function mutations of the Axin-1 gene in another 8% (17–19). Liver-targeted expression of β -catenin transgenes induces hepatomegaly in mice, but at difference with intestinal polyposis or mammary cancer, stabilization of β -catenin appears to be insufficient to cause short term liver cell transformation (20, 21).

A number of downstream target genes of Wnt signaling have been identified in colorectal cancer. These genes play important roles in neoplastic transformation, by affecting growth control and cell cycling (c-Myc, cyclin D1, c-Jun, fra-1, gastrin, WISP-1, ITF-2), cell survival (Id2, MDR1, COX2), or invasion and tumor dissemination (matrilysin, laminin γ 2, VEGF) (22–31). However, although most candidate β -catenin target genes were found to be up-regulated in colon cancer (32), their implication in other tumor types has rarely been investigated and remains to be determined. To better understand the role of activated β -catenin in liver tumorigenesis, we sought to identify genes that are deregulated by overexpression of a dominant stable β -catenin mutant in primary human hepatocytes. These genes were identified by analysis of expression profiles on cDNA arrays, in cells infected with a lentiviral vector that allows expression of transduced genes with high efficiency in nondividing cells (33). The set of differentially expressed genes included previously described targets of β -catenin, as well as known genes not previously linked to the Wnt pathway. The current study demonstrates that the CXC chemokine interleukin-8 (IL-8)¹ is a novel transcriptional target of the β -catenin-

* This work was supported in part by the Association pour la Recherche sur le Cancer (ARC) (to L. L.) and by Grant 5236 from the ARC. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: IL, interleukin; RT, reverse transcriptase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ELISA, enzyme-linked immunosorbent assay; HUVEC, human umbilical vein endothelial cell; LUC, luciferase.

Tcf complex. IL-8 activities on cell growth, motility, and angiogenesis strongly suggest that this chemokine might represent an important downstream effector of the Wnt pathway during developmental and oncogenic processes.

MATERIALS AND METHODS

Plasmid Constructions—A dominant stable β -catenin carrying a mutation at residue 41 (threonine \rightarrow alanine; T41A β -cat) was generated by site-directed mutagenesis of full-length, myc-tagged β -catenin cDNA (a gift of J. Hulsken) and cloned into pcDNA3 as described previously (34). A truncated, transactivation-deficient mutant of β -catenin (Δ N Δ C β -cat) was constructed by amplification of the arm repeat domain (residues 130–680) by PCR with appropriate primers providing an initiation codon and a stop codon and was cloned into pcDNA3. The reporter plasmids pTOP-FLASH and pFOP-FLASH and expression vectors for Tcf4 and myc-tagged dominant negative Δ NTcf4 (4) were kindly provided by H. Clevers. The expression vector for p300 was a gift of Y. Nakatani. IL-8 promoter fragments of different sizes were amplified from human genomic DNA with appropriate sets of primers (available upon request). Mutations of the consensus Tcf/Lef binding site that abolished Tcf binding (AAGATCAAAG \rightarrow AAGGCCAAAG) were introduced in the forward primer of the -193 IL-8 promoter construct. PCR products were cloned into the pCRII-TOPO vector (Invitrogen), and *KpnI-XhoI* fragments were subcloned into the promoterless luciferase vector pGL3-Basic (Promega). All constructs were verified by sequencing.

Primary Cultures of Human Hepatocytes and Fibroblasts—Hepatocytes were prepared from resected normal human livers adjacent to hepatoblastoma or to intrahepatic metastases of breast or colon cancer or from residual graft donor liver fragments. All experimental procedures were conducted in conformity with French laws and regulations and with informed consent of the patients. Hepatocytes were isolated by two-step collagenase perfusion as described previously (35). Briefly, after perfusion with calcium-free HEPES buffer, pH 7.7, and liver tissue digestion with HEPES containing 1 mg/ml collagenase D (Sigma) and 5 mM CaCl₂, the cell suspension was filtered through a 70- μ m mesh cell strainer (BD Biosciences). Cell debris and nonparenchymal cells were partly eliminated by centrifugation at 700 rpm for 1.5 min, and cell viability was assessed by a trypan blue exclusion test. The hepatocyte-enriched fraction was seeded in William's medium on collagen I-coated plates at 7×10^4 cells per cm², in William's medium supplemented with 10 nM insulin, 100 mM triiodothyronine, and 1 mg/ml bovine serum albumin.

Retroviral Vectors and Infection—All constructs were generated using the lentiviral vector pTRIP Δ U3 (36). The pTRIP- β -cat and the pTRIP- Δ N Δ C β -cat constructs were generated by cloning the *BamHI-SalI* fragment containing full-length T41A β -catenin cDNA and the *BglII-XhoI* fragment containing the truncated β -catenin mutant Δ N Δ C β -cat downstream of the cytomegalovirus promoter in the *BamHI-XhoI* sites of pTRIP. The pTRIP-Tcf4 and pTRIP- Δ NTcf4 constructs were generated by cloning *BglII-XhoI* fragments of Tcf4 and Δ NTcf4 cDNAs in the *BamHI-XhoI* sites of pTRIP. To obtain pTRIP-TOP and pTRIP-FOP, a 3130-bp *PvuII* fragment was excised from pTOP-FLASH and pFOP-FLASH and cloned into blunted *MluI-XhoI* sites of the lentiviral vector.

Virions were produced by transient calcium phosphate cotransfection of 293T cells as described previously (33). At 48 h post-transfection, supernatants were treated with DNase and ultracentrifuged, and viral stocks were frozen at -80 °C. The concentration of virion particles was normalized by measuring the p24 capsid protein by ELISA (PerkinElmer Life Sciences). Cells were incubated for 2 h with virions at a concentration of 800 ng of viral p24/ml in one-tenth of the usual volume of William's medium. Fresh medium was then added, and cell were further incubated for 48 h.

RNA Analysis and Hybridization of cDNA Arrays—Total RNA was extracted from primary cells and cell lines using the RNA-PLUS extraction solution (Quantum Biotechnologies). For hybridization of Atlas Plastic Human 8K microarrays (Clontech), ³³P-labeled probes were generated by reverse transcription of 20 μ g of total RNA according to the manufacturer's instructions. After hybridization, filters were scanned at 50- μ m resolution using a Fuji BAS-5000 phosphorimager. Data were processed using Clontech AtlasImage 2.01 software. Intensities were adjusted through a median normalization, and differential expression was considered significant when signal ratio for the same spot was ≥ 2 . For Northern blotting, 10 μ g of total RNA was analyzed by alkaline blot as described previously (37). For RT-PCR, up to 2 μ g of total RNA was reverse-transcribed using Superscript II RT RNase

H-Reverse Transcriptase (Invitrogen) and oligo(dT) primers. Amplifications were carried out in PCR exponential phase (20–35 cycles) to allow comparison among cDNAs synthesized from identical reactions. PCR products were analyzed in 1.5% agarose gels. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as control. Primer sequences are as follows: Keratin-F, 5'-gagtaccaggagg-tgatgaactc-3'; Keratin-R, 5'-AACACAGATCAAGAGCAG-3'; MMP7-F, 5'-GCAGCTATGCGACTCACCG-3'; MMP7-R, 5'-CTGCGCTGAAGTTT-CTATTTC-3'; IL-8-F, 5'-CATGACTTCCAAGCTGGCCG-3'; IL-8-R, 5'-TTTATGAATTCTCAGCCCTC-3'; GAPDH-F, 5'-ACCACAGTCCATGC-CATCAC-3'; GAPDH-R, 5'-TCCACCACCCTGTTGCTGTA-3'.

Cell lines, Transfections, and Reporter Assays—The human kidney cell line 293, the hepatoma cell lines Huh7 and HepG2, and the immortalized human hepatocytes LO2 (38) (a kind gift of P. Pineau), were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. For reporter assays, semi-confluent cells in 6-well plates were transiently transfected by calcium phosphate precipitation with expression vectors for β -catenin, Tcf4, Δ NTcf4, or p300, and 0.1 μ g of different pIL-8-LUC constructs. Luciferase activity was determined 48 h later. All experiments were performed in duplicate and repeated at least three times. A thymidine kinase- β -galactosidase plasmid was cotransfected to normalize luciferase activity for transfection efficiency. However, because p300 was found to activate transcription of this reporter, it could not be used for normalization, and results were confirmed by multiple independent assays. The total amount of transfected DNA was kept constant by adding pcDNA3.

Western Blotting—Cells were lysed in chilled lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na₃VO₄, 1 mM NaF) supplemented with protease inhibitors (Roche Molecular Biochemicals). Whole cell extracts were resolved in 8–16% Tris-HCl polyacrylamide gels (Novex) and transferred to Hybond-C extra membranes (Amersham Biosciences). Western blots were probed with a monoclonal anti- β -catenin antibody (Transduction Laboratories) or a polyclonal anti-IL-8 antibody (BIOSOURCE), followed by secondary antibodies. Immunoreactive proteins were visualized using protocols and reagents of the Western CDP-star kit (PerkinElmer Life Sciences).

Cytokine ELISA—LO2 cells were plated at 10^4 cells/ml, infected with different lentiviral vectors as indicated, and supernatants were collected at different times and frozen immediately. IL-8 ELISA was performed on 1-ml aliquots as described previously (39), using a monoclonal anti-human IL-8 antibody provided by J.-C. Mazié (Hybridolab, Institut Pasteur) and a rabbit polyclonal anti-IL-8 antibody kindly provided by Dr. N. Vita (Sanofi Recherche, Labège, France).

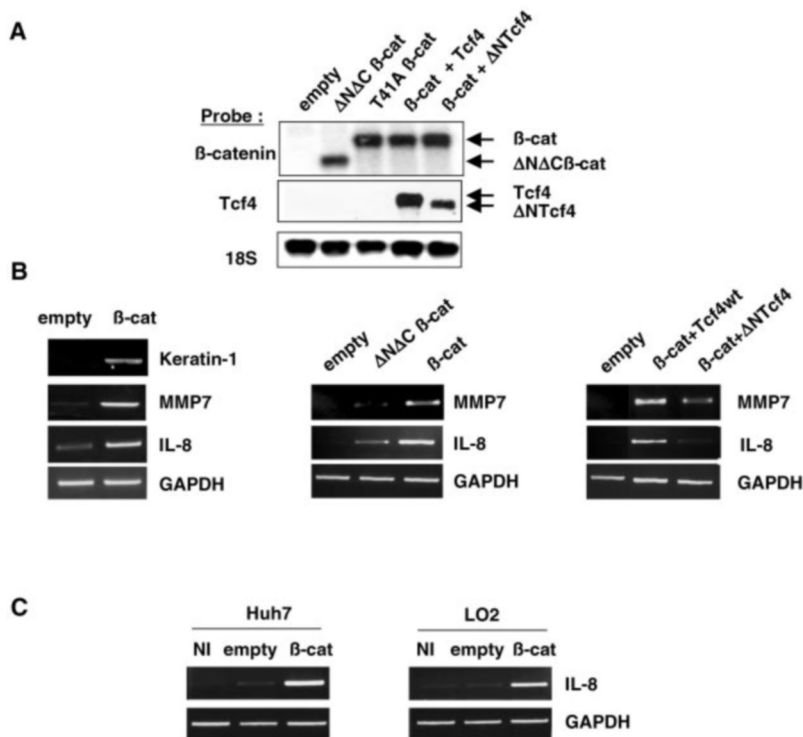
Endothelial Cell Culture and Migration Assays—Human umbilical vein endothelial cells (HUVECs) (Clonetics; BioWhittaker) were propagated through passage 6 in MCDB131 medium (Invitrogen) supplemented with 2 mM Glutamax (Invitrogen), 12% fetal calf serum, 10 units/ml porcine heparin (Sigma), 10 ng/ml hu-EGF (Peprotech Inc.), 35 μ g/ml endothelial cell growth supplement (BD Biosciences), and 1 μ g/ml hydrocortisone (Sigma). Subconfluent HUVECs were starved for 2–3 h in M199 medium containing 2% fetal calf serum and 1 μ M Calcein-AM (Molecular Probes) for cell labeling. Cells were trypsinized, pelleted, and resuspended in M199 medium containing 0.1% fatty acid-free bovine serum albumin (I. D. Bio, Limoges, France). Cells (5×10^4 per well) were placed in the upper chambers of 8- μ m cell culture inserts (Falcon HTS Fluoroblock; BD Biosciences) coated with 50 μ g/ml collagen I. The lower wells contained conditioned medium from LO2 cells infected with different pTRIP constructs as indicated. For control, we used either M199 with 0.1% bovine serum albumin (background control) or MCDB 131 containing 8% fetal calf serum and 30% conditioned medium from differentiating primary human erythroblasts (control chemoattractant medium) (40). IL-8-neutralizing antibody or control goat IgG (1 μ g/well) was included in lower wells 30 min before migration was monitored. After incubation for 2 h at 37 °C, cells on the upper side of the filters were washed off, and cells that had migrated through the filters were fixed in formalin, stained with propidium iodide (2 μ g/ml in phosphate-buffered saline, overnight at 4 °C), and counted under a fluorescent microscope. At least ten random fields per well at 32 \times magnification were counted for each experiment.

RESULTS

Identification of Downstream Target Genes of β -Catenin in Primary Human Hepatocytes—To identify genes whose expression is regulated by β -catenin-Tcf in the liver context, we analyzed differentially expressed genes by microarray profiling in primary human hepatocytes after β -catenin gene transfer. Human hepatocytes at 24 h post-plating were infected with the

FIG. 1. IL-8 is up-regulated by β -catenin-Tcf4 in hepatocytes and hepatoma cell lines.

A, primary human hepatocytes at 24 h post-plating were infected with 800 ng/ml of either pTRIP- Δ N Δ C β -cat encoding the arm repeat domain of β -catenin or pTRIP- β -cat, alone or along with pTRIP-Tcf4 or pTRIP- Δ NTcf4. Total RNA was extracted from each culture 48 h after infection, and 10 μ g of RNA was analyzed by Northern blotting with β -catenin and Tcf4 probes. An 18 S cDNA probe served as control for equal loading. **B**, β -catenin-driven up-regulation of the candidate target gene IL-8 and the known Wnt-responsive genes MMP7 and keratin-1 was confirmed by RT-PCR, using 2 μ g of RNA from infected hepatocytes. The transactivation-deficient Δ N Δ C β -catenin had only a modest effect, and the dominant negative Tcf4 strongly down-regulated IL-8 and MMP7 expression. **C**, IL-8 expression was analyzed by RT-PCR in the hepatoma cell lines Huh7 and LO2 infected or not with the empty vector and with pTRIP- β -cat. Expression of GAPDH served as control. NI, not infected.



lentiviral vector pTRIP Δ U3 (36) or with a recombinant vector (pTRIP- β -cat) expressing the stable dominant T41A β -catenin under control of the cytomegalovirus promoter. The TRIP vector was chosen, because it allows high transduction efficiency in differentiated, nondividing cells (33). Our preliminary experiments with pTRIP- β -cat indicated that over 90% of hepatocytes abundantly expressed β -catenin in the nucleus. Moreover, co-infections with pTRIP- β -cat and vectors expressing the luciferase reporter under control of consensus Tcf binding motifs (pTRIP-TOP) or its mutated, unresponsive version (pTRIP-FOP) indicated that activation of Tcf-mediated transcription reached maximal values at 48 h (data not shown). This time was therefore selected in microarray experiments. To discriminate direct transcriptional targets of the β -catenin-Tcf complex among deregulated genes, we also infected hepatocytes with an expression vector for truncated β -catenin retaining the central arm repeat region and devoid of transactivation activity (pTRIP- Δ N Δ C β -cat). As shown in Fig. 1A, both full-length and truncated β -catenin were highly expressed in infected hepatocytes.

cDNA probes were generated from the RNA of primary human hepatocytes infected with pTRIP- β -cat, pTRIP- Δ N Δ C β -cat, and empty vector. These probes were used for differential hybridization with a cDNA array (Atlas 8K; Clontech) containing 8,000 sequence-verified, known human genes. Around 1,200 genes were detectably expressed in cultured hepatocytes, including liver markers such as hepatic arginase and hepatic lipase, consistent with the observed persistence of hepatocyte-like morphology during short term culture. We have compared the expression profiles between hepatocytes infected with pTRIP- β -cat and the empty vector and identified 200 genes that were differentially expressed by 2-fold or higher, including 57 genes that were up-regulated. In a second step, expression profiles were compared between cells expressing T41A β -catenin and those expressing the transactivation-deficient Δ N Δ C β -catenin. Forty-two of the 57 genes were still expressed differentially, suggesting that their up-regulation was dependent upon β -catenin transactivation activity. Among these genes, we noted known Wnt-responsive genes such as matrilysin (MMP7)

TABLE I

Selected genes differentially expressed in cDNA array screens

Expression profiles were compared between hepatocytes infected with pTRIP- β -cat or the empty vector and between hepatocytes infected with pTRIP vectors expressing dominant stable β -catenin or the truncated, inactive Δ N Δ C β -catenin. The ratios of signal intensities obtained for four genes up-regulated by β -catenin are shown. β -Catenin data are presented as control. Note that Δ N Δ C β -catenin did not hybridize with C-terminal β -catenin cDNA sequences spotted on the array.

| Genes | Ratios | | GenBank™ Accession No. |
|------------------|--------------------|--|------------------------|
| | β -Cat/Empty | β -Cat/ Δ N Δ C β -cat | |
| MMP7 | 3 | 2 | Z11887 |
| Keratin-1 basic | 6.2 | 6.2 | NM_002281 |
| CTGF | 2.75 | 3.3 | NM_001901 |
| Interleukin-8 | 4.6 | 2.6 | Y00787 |
| β -Catenin | 15 | 15 | Z19054 |

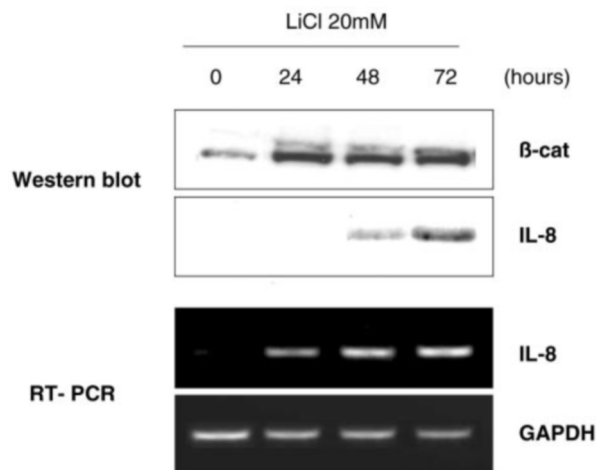


FIG. 2. LiCl induces IL-8 expression. LO2 cells were incubated with 20 mM LiCl for different times as indicated. Total lysates were prepared, and expression of β -catenin and IL-8 proteins was assessed by Western blot analysis using 30 μ g of cell lysate per lane (upper panel). Total RNA was isolated from cells harvested at the same time points, and IL-8 mRNA was analyzed by RT-PCR using GAPDH as a control (lower panel).

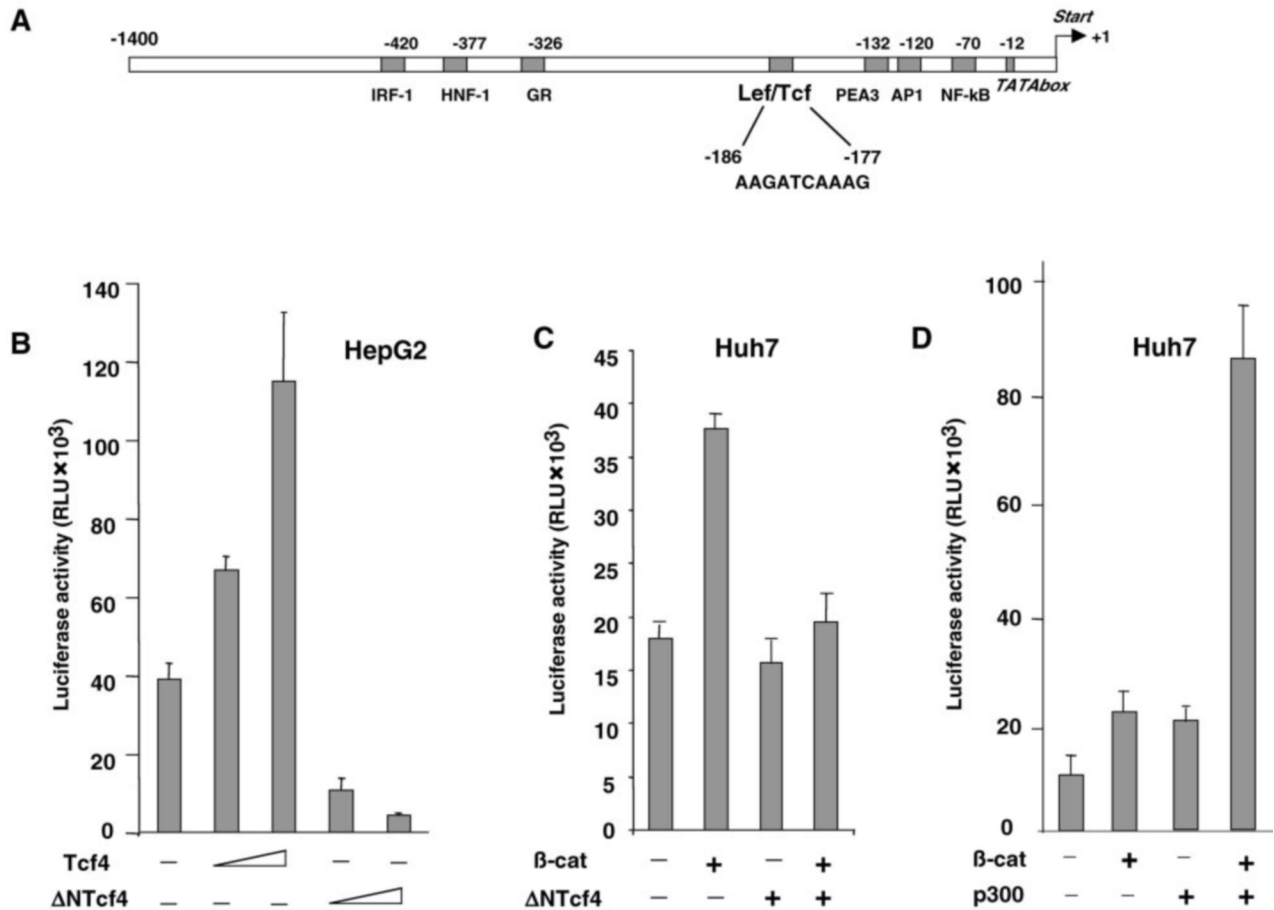


FIG. 3. The IL-8 promoter is activated by β -catenin-Tcf4. A, schematic representation of the IL-8 promoter and binding sites for multiple nuclear factors. A putative Tcf/Lef binding element is localized at position -186 bp from the transcription start site. B, HepG2 cells containing a constitutively activated β -catenin allele were cotransfected with $0.1 \mu\text{g}$ of a reporter plasmid containing 1.4 kb of the IL-8 promoter (1400-IL-8-LUC) and 1 – $4 \mu\text{g}$ of Tcf4 or Δ NTcf4 expression vector and harvested 48 h after transfection. Total amounts of plasmid DNA were kept constant by adding the empty pcDNA3 vector. All experiments were performed in duplicate and repeated at least three times. Means \pm S.D. are presented. C, Huh7 cells containing normal endogenous β -catenin were transiently transfected with $0.1 \mu\text{g}$ of 1400-IL-8-LUC, along with $1 \mu\text{g}$ of T41A β -catenin and/or $3 \mu\text{g}$ of Δ NTcf4 expression vector. D, IL-8 promoter transactivation by the p300 coactivator. Huh7 cells were transfected with the 1400-IL-8-LUC reporter ($0.1 \mu\text{g}$), along with expression vectors for β -catenin ($0.5 \mu\text{g}$) and/or p300 ($3 \mu\text{g}$).

and basic keratin-1 genes, as well as a member of the WISP family, the connective tissue growth factor (Table I), whereas cyclin D1 expression remained undetectable in all settings.

Up-regulation of IL-8 by β -Catenin-Tcf4—Search for consensus Tcf binding sites in the promoters of candidate genes revealed the presence of AAGATCAAAG sequences at position -186 to -177 in the IL-8 promoter. IL-8 mRNA was enhanced 4.6-fold by β -catenin in the first cDNA array screen and was still higher in the second screen when cells expressing transcriptionally active β -catenin were compared with those expressing transcription-defective β -catenin (Table I). Differential expression of IL-8 in β -catenin-expressing cells was confirmed by semi-quantitative RT-PCR analysis in two independent primary hepatocyte cultures, as also found for MMP7 and keratin-1 genes (Fig. 1B). Furthermore, IL-8 mRNA levels were higher in hepatocytes expressing constitutively active β -catenin than in those expressing Δ N Δ C β -catenin. To assess whether IL-8 transcriptional activation was Tcf4-dependent, we performed co-infections with pTRIP- β -cat and vectors expressing either wild type Tcf4 (pTRIP-Tcf4) or a dominant negative mutant devoid of β -catenin binding domain (pTRIP- Δ NTcf4). Efficient expression of wild type and mutant Tcf4 in hepatocytes was verified by Northern blotting (Fig. 1A). The Δ NTcf4 mutant strongly inhibited β -catenin-induced IL-8 expression (Fig. 1B). Similar data were obtained in the well

differentiated hepatic cell lines Huh7 and LO2, in which the β -catenin pathway is not activated constitutively (Fig. 1C).

Up-regulation of IL-8 was also evidenced, both at mRNA and protein levels, in LO2 cells after treatment with LiCl 20 mM (Fig. 2). LiCl is an inhibitor of GSK3 β that induces strong accumulation of dephosphorylated β -catenin and increased activity of the synthetic Tcf-dependent luciferase reporter TOPFLASH (7). In our experiments, IL-8 mRNA was induced at 24 h of LiCl treatment, when the levels of β -catenin were increased markedly, whereas IL-8 protein was detectable at 48 h, strongly suggesting direct activation of IL-8 expression by β -catenin (Fig. 2). By contrast, we repeatedly failed to detect any change in IL-8 mRNA levels after transduction of β -catenin into primary human fibroblasts, although efficient expression of β -catenin from the pTRIP vector in these cells was verified by Northern blotting (data not shown). This suggests that β -catenin-driven induction of IL-8 expression might be dependent on cellular context.

Transactivation of the IL-8 Promoter by the β -Catenin-Tcf4 Complex—The presence of a consensus Tcf/Lef binding motif in the IL-8 promoter (Fig. 3A) prompted us to examine the effects of β -catenin and Tcf4 expression on IL-8 promoter activity. Two hepatoma cell lines were used, Huh7, in which normal β -catenin is localized to the cell membrane, and HepG2, which exhibits nuclear accumulation of N-terminally deleted β -catenin

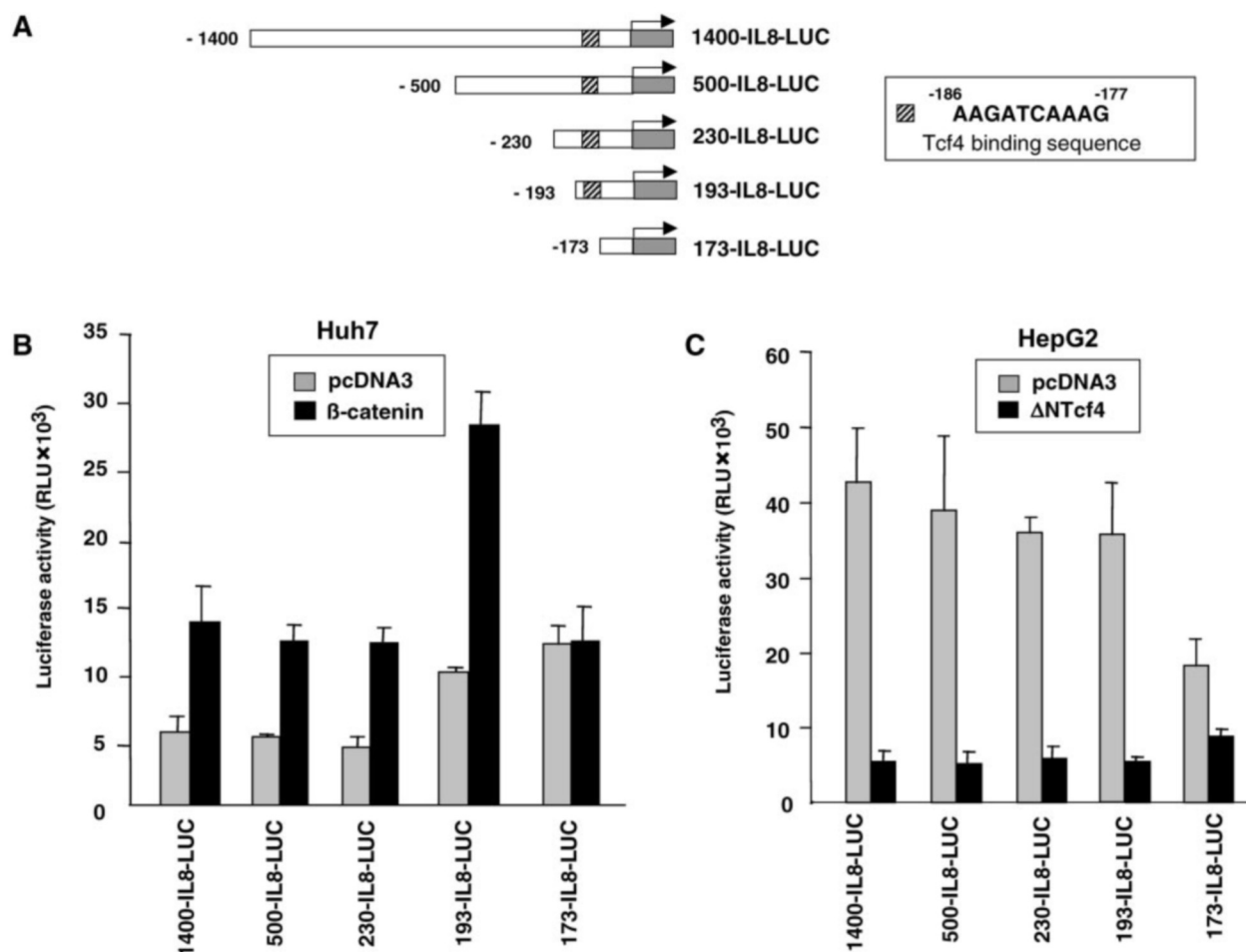


FIG. 4. Identification of β -catenin-responsive element by deletion analysis of the IL-8 promoter. *A*, successive deletion constructs of the 1.4-kb IL-8 promoter (500, 230, 193, and 173 bp) in the pGL3 luciferase reporter plasmid. *B*, these constructs were transfected into Huh7 cells, along with a β -catenin expression vector or the empty vector as described in the legend for Fig. 3. *C*, HepG2 cells were cotransfected with 0.1 μ g of the indicated IL-8 promoter constructs and 4 μ g of Δ NTcf4 expression vector or empty pCDNA3 vector.

(17). A 1.4-kb IL-8 promoter-luciferase construct (1400-IL-8-LUC) was transfected into Huh7 and HepG2 cells, along with different β -catenin and Tcf4 expression vectors. In HepG2 cells, transfection of increasing amounts of Tcf4 further stimulated the basal IL-8 promoter activity up to 3-fold, whereas Δ NTcf4 reduced the basal activity up to 9-fold in a dose-dependent manner (Fig. 3*B*). In Huh7 cells, the IL-8 promoter was activated 2-fold by cotransfection with β -catenin, and Δ NTcf4 inhibited β -catenin-driven transactivation (Fig. 3*C*).

Because interaction of the cAMP-response element-binding protein-binding protein and p300 coactivators with β -catenin has been shown to potentiate transcriptional activation of some Wnt-responsive genes (11), we next tested whether p300 cooperates with β -catenin in IL-8 promoter activation. As shown in Fig. 3*D*, coexpression of p300 and β -catenin, along with the IL-8 promoter construct in Huh7 cells, resulted in an 8-fold increase in luciferase activity, whereas p300 had little activity in the absence of β -catenin. Similar data were obtained in 293 cells. Thus, p300 is required for robust β -catenin responsiveness of the IL-8 promoter.

To determine the functional significance of consensus Tcf/Lef binding sequences at nucleotides -186 to -177, we first employed a series of 5' promoter deletion constructs (Fig. 4*A*). In Huh7 cells, a 2–3-fold induction in the IL-8 promoter activity by β -catenin was conserved for the 500-, 230-, and 193-bp proximal promoter fragments, but β -catenin had no effect on the -173 construct, in which the putative site was deleted (Fig. 4*B*). Conversely, in HepG2 cells, similar basal activity was

observed for the 1.4-kb to 193-bp promoter fragments, whereas the -173 construct showed a 2-fold lower activity (Fig. 4*C*). Furthermore, basal activity of the 1.4-kb to 193-bp promoter constructs was 8- to 10-fold down-regulated by Δ NTcf4 in these cells, but the 173-bp fragment showed only a 2-fold decrease, which is consistent with positive regulation of the IL-8 promoter by the β -catenin-Tcf complex. The functional significance of the consensus Tcf/Lef binding motif was assessed further by introducing point mutations in the context of the -193 IL-8 promoter (Fig. 5*A*). Mutation of the Tcf/Lef binding motif abolished transactivation of this construct by β -catenin in Huh7 cells (Fig. 5*B*). In HepG2 cells, basal activity of the mutated -193 construct was reduced by 3-fold, indicating that the β -catenin-Tcf4 complex contributes to a significant extent to constitutive IL-8 expression in these cells. Accordingly, the inhibitory effect of Δ NTcf4 on 193mut-IL8-LUC activity was decreased strongly compared with the corresponding wild type reporter (Fig. 5*C*). These data show that induction of IL-8 by β -catenin is controlled at the transcription level and depends on a single Tcf/Lef binding site.

β -Catenin Induces Secretion of IL-8 with Chemoattractant Activity—It has been shown that the IL-8 chemokine is involved in angiogenesis (41). To assess the biological significance of IL-8 induction by β -catenin, we thought to determine whether overexpression of β -catenin was associated with secretion of functionally active IL-8. LO2 cells expressing low, barely detectable levels of IL-8 were infected with pTRIP- β -cat, pTRIP- Δ NTcf4, or empty vector. Cells were washed, fresh me-

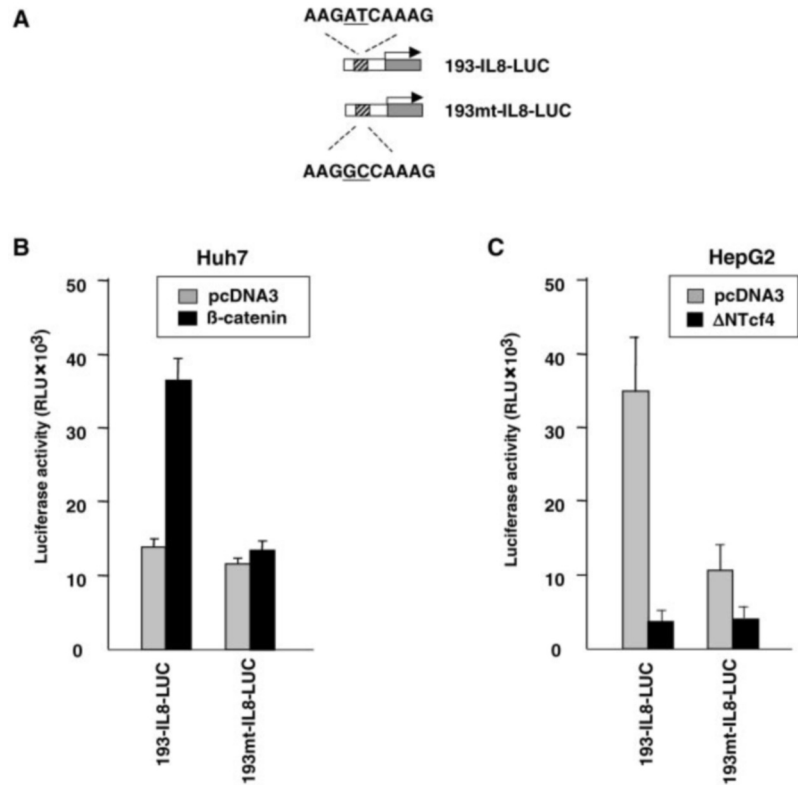


FIG. 5. The Tcf/Lef binding sequence at position -186 is crucial for activation of the IL-8 promoter by β -catenin-Tcf4. A, point mutations known to abolish Tcf binding were introduced into the Tcf/Lef recognition site in the context of the -193 IL-8 promoter. B, Huh7 cells were transfected with wild type or mutated 193-IL8-LUC reporter, along with β -catenin or empty vector as indicated in the legend for Fig. 3. C, HepG2 cells were cotransfected with wild type or mutant 193-IL8-LUC reporter and dominant negative Tcf4 or the empty vector.

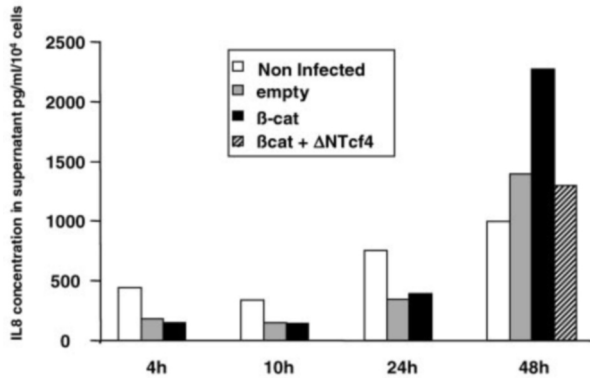


FIG. 6. β -Catenin induces secretion of IL-8. LO2 cells were plated at 10^4 cells/ml in 12-well plates and infected with TRIP- β -cat alone or along with TRIP- Δ NTcf4. Noninfected cells and cells infected with the empty vector served as controls. Fresh medium was added 2 h post-infection, and supernatant was collected at different times as indicated for ELISA tests of IL-8 production.

dium was added 2 h later, and conditioned media were analyzed by ELISA at different times. Significant secretion of IL-8 was seen at 48 h in β -catenin-expressing cells but not in cells infected with empty vector or in the presence of dominant negative Tcf4 (Fig. 6). The level of IL-8 protein increased further after 48 h because of persistent expression of β -catenin transduced by the lentiviral vector (data not shown). It has been shown that the IL-8 chemokine can induce migration of cells expressing the CXC chemokine receptors, notably endothelial cells (42). To determine whether β -catenin-driven IL-8 release might exert chemoattractant effects on neighboring cells, migration analysis was performed using HUVECs and chamber assembly. Migration of HUVECs was stimulated by conditioned media from untreated HepG2 cells or from LO2 cells infected with pTRIP- β -cat but not from LO2 cells infected with the empty vector. Importantly, stimulation levels decreased to background when IL-8-neutralizing antibody

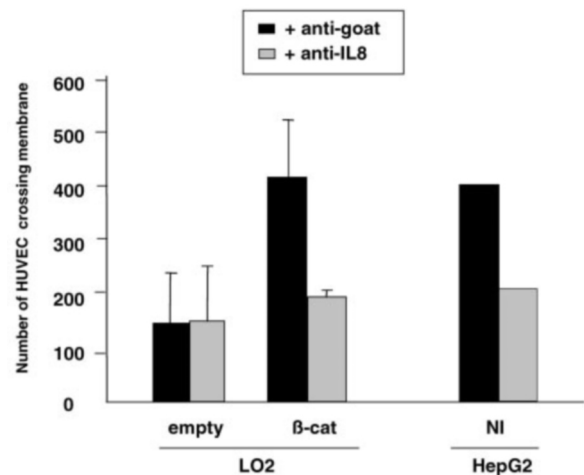


FIG. 7. β -Catenin has chemoattractant effect through the induction of IL-8. LO2 cells (2×10^5 cells per well in 6-well plates) were infected with pTRIP- β -cat or empty vector, supernatants were discarded 24 h post-infection, and cells were incubated in fresh medium for 48 h. Migration assays were performed using HUVECs and chambers of 8- μ m cell culture inserts in the presence of 48-h conditioned medium from infected LO2 cells or from noninfected (NI) HepG2 cells. Neutralizing antibody against IL-8 (1 μ g/well) was added to determine the functional contribution of IL-8 on cell migration. Nonspecific goat antibody was used to control the specificity of the inhibition. At least ten random fields per well were counted for each experiment. Data were gathered from three independent assays.

was added prior to the migration test (Fig. 7). These data show that β -catenin-induced migration of endothelial cells is mediated by IL-8.

DISCUSSION

In this study, we have identified the CXC chemokine IL-8 as a gene up-regulated by β -catenin by using microarray technology and primary human hepatocytes that expressed stabilized or transcription-defective β -catenin. We show that endogenous

IL-8 mRNA is induced in hepatocytes and hepatoma cell lines by ectopic overexpression of dominant stable β -catenin, as well as by LiCl treatment, which inactivates GSK3 β and therefore stabilizes wild type β -catenin. This regulation occurs at the transcription level, because the IL-8 promoter responded to β -catenin in Huh7 cells expressing low wild type β -catenin and to Tcf4 in HepG2 cells expressing elevated mutant β -catenin whereas dominant negative Tcf4 inhibited these effects. Our data demonstrate that a single consensus Tcf/Lef binding sequence located 186 bp from the transcriptional start site is critical for β -catenin responsiveness, thus identifying IL-8 as a direct target of β -catenin-Tcf4. Although activation levels triggered by β -catenin alone were weak, the p300 coactivator cooperated strongly with β -catenin to specifically activate the IL-8 promoter. Thus, in this promoter context, β -catenin recruits p300 as a coactivator for efficient transactivation, as reported previously for the *siamois* promoter (11).

IL-8 is constitutively up-regulated in a variety of human cancers such as melanoma and lung, gastric, prostate, and bladder cancers (43–46). Interestingly, up-regulation of IL-8 has been linked recently to β -catenin activation based on microarray analysis of differentially expressed genes between normal and neoplastic colon.² In hepatocellular carcinoma, overexpression of IL-8 has also been observed in about half of the cases, and tumor cells were shown to represent the major source of IL-8 production (47). Hepatocellular carcinomas develop on a background of chronic hepatitis or cirrhosis, in which viral and inflammatory factors trigger potent IL-8 induction (48). Moreover, the IL-8 gene has been identified as a target of hepatocyte growth factor and insulin-like growth factor-1 (49, 50), and activation of either of these pathways also leads to nuclear activation of β -catenin (51, 52). Therefore, IL-8 expression levels in cancer cells might be modulated by a complex interplay of signaling pathways.

The pleiotropic activities of IL-8 as a mitogenic, motogenic, and angiogenic factor (53) imply that the chemokine might play important roles in development and tumorigenesis. Although the role of IL-8 at developmental stages remains to be documented, it has been shown that IL-8 acts as an autocrine growth factor for a variety of cancer cell lines. The IL-8 receptors CXCR1 and CXCR2 are expressed in hepatoma cells (54), and antisense oligonucleotides or IL-8-neutralizing antibodies can suppress growth of various cancer cells (46). We found recently that exposure of hepatoma cells to IL-8 activates the mitogen-activated protein kinase pathway and the phosphorylation of extracellular signal-regulated kinase 1/2, which are important mediators of growth signals from cell surface receptors to the nucleus.³

A major role of IL-8 in tumor angiogenesis has been demonstrated by functional studies showing its ability to induce endothelial cell chemotaxis and neovascularization (41, 44). In this study, we show that β -catenin-expressing cells induce migration of human vascular endothelial cells. IL-8-neutralizing antibodies abolished this effect, indicating that this mitogenic activity is mediated directly by IL-8 in hepatoma cells. β -Catenin might influence angiogenesis by activating a combination of several proangiogenic factors, such as vascular endothelial growth factor, which was also identified recently as a target of the Wnt/ β -catenin pathways (30). Importantly, IL-8 is also involved in tumor invasion and metastasis (46, 47). It has been reported that IL-8 increases the expression of metalloproteinases MMP2 and MMP9 in human melanocytes (45). Matrilysin (MMP7), which we found up-regulated by β -catenin in hepato-

cytes, and laminin γ 2 are other Wnt target genes implicated in tumor invasiveness. In colon cancer, overexpression of β -catenin and its target genes at invasive tumor fronts has been correlated with increased risk of tumor recurrence and poor outcome (55, 56). The present study, linking IL-8 to the Wnt pathway, further emphasizes the role of tumor microenvironment in cancer progression and provides new insight into β -catenin functions in vasculogenesis and angiogenesis.

Acknowledgments—We thank Drs. H. Clevers, J. Hulsken, and Y. Nakatani for providing plasmids and Julien Pothlichet for help as a graduate student. We are grateful to Pierre Tiollais for constant interest in this work.

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