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Bcl-2 overexpression in PhIP-induced colon tumors: cloning of the rat *Bcl-2* promoter and characterization of a pathway involving β-catenin, c-Myc and E2F1

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Abstract

β-Catenin/T-cell factor (Tcf) signaling is constitutively active in the majority of human colorectal cancers, and there are accompanying changes in Bcl-2 expression. Similarly, 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP)-induced colon tumors in the rat have increased *β*-catenin and elevated Bcl-2. To examine the possible direct transcriptional regulation of rat *Bcl-2* by *β*-catenin/Tcf, we cloned and characterized the corresponding promoter region and found 70.1% similarity with its human counterpart, *BCL2*. *Bcl-2* promoter activity was increased in response to LiCl and exogenous *β*-catenin, including oncogenic mutants of *β*-catenin found in PhIP-induced colon tumors. Protein/DNA arrays identified E2F1, but not *β*-catenin/Tcf, as interacting most strongly with the rat *Bcl-2* promoter. Exogenous E2F1 increased the promoter activity of rat *Bcl-2*, except in mutants lacking the E2F1 sites. As expected, *β*-catenin induced its downstream target c-Myc, as well as E2F1 and Bcl-2, and this was blocked by siRNA to c-Myc or E2F1. These findings suggest an indirect pathway for Bcl-2 overexpression in PhIP-induced colon tumors involving *β*-catenin, c-Myc and E2F1.

Keywords

β-catenin; Bcl-2; c-Myc; E2F1; wnt signaling; colorectal cancer

Introduction

Most human colorectal cancers have mutations in the adenomatous polyposis coli (*APC*) gene or *β*-catenin gene (*CTNNB1*), leading to increased *β*-catenin protein expression and activation of downstream *β*-catenin/T-cell factor (Tcf) target genes (Behrens, 2005). These cancers also exhibit marked changes in the expression of Bcl-2 family proteins (Bedi *et al*., 1995).

Similar findings have been reported in the colon tumors from animals treated with 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine (PhIP) and 2-amino-3-methylimidazo(4,5-*f*) quinoline (IQ), which are dietary agents that the US National Toxicology Program has classified as ‘reasonably anticipated to be human carcinogens’. Indeed, there are a number of similarities between human and rat colon tumors with respect to *β*-catenin/Tcf signaling and the expression of Bcl-2 family proteins. First, as in the human situation, PhIP-and IQ-induced colon tumors in the rat contain mutations in *Apc* or *Ctnnb1*, but not in both of these genes (Dashwood *et al*., 1998). Second, these mutations stabilize *β*-catenin through inhibition of

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phosphorylation, ubiquitination and proteasome degradation, leading to increased expression of *β*-catenin protein (Al-Fageeh *et al*., 2004). Third, *β*-catenin/Tcf target genes frequently are overexpressed, including *c-Myc* (Blum *et al*., 2001, 2003). Fourth, during the progression from normal colonic mucosa to adenoma and carcinoma there is an increase in anti-apoptotic Bcl-2 protein and loss of proapoptotic Bax (Hayashi *et al*., 1996).

These findings allowed us to ask whether changes in apoptosis-related proteins, and specifically Bcl-2, might be directly related to *β*-catenin/Tcf signaling. Cloning and characterization studies ruled out the rat *Bcl-2* gene as a direct target of *β*-catenin, but c-Myc and the transcription factor E2F1 were identified as intermediates in the upregulation of rat *Bcl-2*.

Results

The ratio of Bcl-2/Bax mRNA is increased in PhIP-induced colon tumors containing *β*-catenin mutations

Previous work showed elevated Bcl-2 and decreased Bax protein levels in heterocyclic amine-induced colon tumors (Hayashi *et al*., 1996); thus, we first examined the expression of *Bcl-2* and *Bax* mRNA in six PhIP-induced colon tumors. Results are summarized in Table 1 in terms of the *Bcl-2*/*Bax* ratio, together with the mutation status of *β*-catenin, as determined by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) analysis. Codons 32 and 34 of *Ctnnb1* are known 'hot spots' for heterocyclic amineinduced mutation (Dashwood *et al*., 1998; Blum *et al*., 2003), and three PhIP-induced colon tumors had *β*-catenin G*G*A→GAA (G34E) or G*G*A→GTA (G34 V) mutations. Although such mutations do not substitute critical Ser/Thr residues directly, they nonetheless stabilize the *β*-catenin protein by interfering with the relative extent of Ser33 phosphorylation by glycogen synthase kinase-3*β* (GSK-3*β*) and subsequent ubiquitination/proteasome degradation (Al-Fageeh *et al*., 2004). No *β*-catenin mutations were detected in three other colon tumors (Table 1). Interestingly, tumors with *β*-catenin mutations had an 8-fold increase in *Bcl-2* versus *Bax* expression, whereas tumors that were wild-type for *β*-catenin had no marked changes in *Bcl-2* or *Bax*. This suggested that *β*-catenin might upregulate *Bcl-2* gene expression through direct or indirect mechanisms.

Cloning andcharact erization of the rat *Bcl-2* 5′-flanking sequence

To investigate whether *Bcl-2* might be a direct *β*-catenin/Tcf target gene in the rat, we cloned a 1745 bp fragment of the 5′-flanking region of rat *Bcl-2*, also containing part of exon 1 (see GenBank accession no. AF531426). Analysis using MatInspector (Genomatix) revealed putative DNA-binding sites for E2F1, NF*κ*B, AP-2, SP1, c-Myb, SMAD, GATA, PAX3 and other transcription factors. Interestingly, four putative Tcf binding sites also were predicted.

In 5′-RACE experiments, fresh poly(A)$^+$RNA (Ambion, Austin, TX, USA) consistently generated a single 260-bp product and no larger or smaller fragments, suggesting only full-length poly(A)$^+$RNAs were amplified (Figure 1a). Moreover, following gel purification and sequence analysis (Figure 1b), the transcription start site was readily assigned to a C nucleotide 231 bp upstream of the translation start site ATG, with a similar genomic organization as human *Bcl-2* (Figure 1e). Indeed, h*Bcl-2* and r*Bcl-2* share 70.1% similarity, and the core promoter region of r*Bcl-2* has 85% identity with h*Bcl-2*, containing several GC boxes, and CAAT and TATA box motifs located close to the AUG translation start site, as in h*Bcl-2* (Harigai *et al*., 1996).

In human embryonic kidney (HEK) 293 cells (Figure 1c), transient transfection of a reporter fragment containing −1945 to −906 of rat *Bcl-2* (designated hereafter as 'P1') showed 10 times higher promoter activity than the fragment between −905 and −1 (referred to hereafter as 'P2').

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Sequential deletion analysis showed that deletion at position −1201 (F7-1) or shorter resulted in significant loss of *Bcl-2* promoter activity (Figure 1d). However, F8-1, F9-1 and F10-2 had high promoter activity, indicating that the core promoter is located between −1201 and −1489 bp. Interestingly, F10-1 had lower promoter activity than F9-1 and F10-2, suggesting that negative regulatory element(s) might exist at each end of the *Bcl-2* promoter.

Identification of transcription factors bound to the rat *Bcl-2* promoter

Nuclear proteins bound to the rat *Bcl-2* promoter were purified by DNA pull-downs (Zeng *et al*., 2003), and they were identified using protein/DNA arrays, as reported before (Li *et al*., 2004). In Array I (Figure 2a), strong signals were obtained for E2F1 (dotted box), NF*κ*B, MEF1, CBF, USF1, c-Myb, NF-1, Pax5 and Smad3/4. There also were signals for AP-1, AP-2, p53, GATA and CREB. In Array II, strong signals were detected for GATA3, ISRE, PARP, RREB2, Pax3 and ZIC (Figure 2b). However, no Tcf signal was observed (Figure 2b, box), even though four putative Tcf sites were predicted using MatInspector software.

Subsequently, mobility-shift assays were performed with ^{32}P-labeled oligos containing one of the four putative Tcf sites, *in vitro* translated Tcf4 (or Lef1), and nuclear extracts from HEK 293 cells. Promising bands initially were observed, but excess cold oligo failed to produce the expected competition, and no supershift was seen with Tcf4 or Lef1 antibodies (data not shown). Taken together with the protein/DNA analyses (Figure 2b), we concluded that the putative Tcf sites in rat *Bcl-2* were not authentic.

E2F1 is a transcriptional activator of rat *Bcl-2*

Because no authentic Tcf sites were found, 10 different transcription factors with affinity for the rat *Bcl-2* promoter in protein/DNA arrays (Figure 2) were tested for their effects on *Bcl2*-P1. A striking response was seen with E2F1, which increased promoter activity >10-fold (Figure 3a). Reporter assays were performed with deletion constructs containing two, one, or zero E2F sites in the rat *Bcl-2* promoter (Figure 3b). Exogenous E2F1 increased reporter activities 7- to 11-fold with constructs containing both E2F sites, but there was no effect with constructs containing one or no E2F sites. Site-directed mutagenesis targeted at both E2F sites completely abolished the induction of *Bcl-2* promoter activity by exogenous *β*-catenin, and the response was markedly attenuated by mutation of site 1, but not site 2 (Figure 3c). Thus, site 1 appears to be more important for *β*-catenin- or E2F1-induced activation of the *Bcl-2* promoter.

β-Catenin upregulates *Bcl-2* promoter activity

Although *Bcl-2* was excluded as a direct target of *β*-catenin, owing to the lack of authentic Tcf sites (see above), we observed induction of *Bcl-2*-P1 reporter activity after forced expression of *β*-catenin. Thus, in HEK293 cells, overexpression of wild-type *β*-catenin by transient transfection increased the reporter activity twofold (Figure 4a), and LiCl, a well-known inhibitor of GSK-3*β* that stabilizes endogenous *β*-catenin (Aberle *et al*., 1997; Al-Fageeh *et al*., 2004), also increased *Bcl-2* promoter activity. Immunoblotting of whole cell lysates confirmed that *β*-catenin and Bcl-2 proteins were increased by exogenous *β*-catenin and LiCl treatment (Figure 4b). Rather than using other means to increase endogenous wild-type *β*-catenin, such as soluble Wnt3a (Park *et al*., 2006), we examined oncogenic mutants found in PhIP-induced colon tumors, namely D32N, G34E and Δ45 (Figure 4c). Each *β*-catenin mutant activated *Bcl-2* reporter activity more effectively than wild-type *β*-catenin, and there was a concomitant increase in Bcl-2 and *β*-catenin protein expression in cell lysates.

β-Catenin upregulates c-Myc, E2F1 andhence Bcl-2

Because c-Myc is a well-known direct target of *β*-catenin/Tcf signaling (He *et al*., 1998), we next used siRNA to knockdown c-Myc. Overexpression of *β*-catenin induced rat *Bcl-2*

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promoter activity (Figure 5a, upper panel), and increased protein expression of c-Myc, E2F1 and Bcl-2 (Figure 5a, lower panel). Reduction of c-Myc by siRNA blocked *β*-caten-independent induction of rat *Bcl-2* promoter activity, as well as E2F1 and Bcl-2 protein expression. These data confirmed that c-Myc is not only a *β*-catenin/Tcf target, but also an E2F1 activator. Finally, using siRNA to knockdown E2F1 (Figure 5b), there was a corresponding attenuation of *Bcl-2* promoter activity and Bcl-2 protein expression, with more cells undergoing apoptosis, as evidenced by an increased in cleaved (active) caspase-3.

Discussion

Elevated expression of *β*-catenin has been detected in colorectal and other cancers, resulting in constitutive activation of numerous *β*-catenin/Tcf target genes (Behrens, 2005). We reported on the high frequency of *β*-catenin mutations in PhIP- and IQ-induced rat colon tumors (Dashwood *et al*., 1998), the overexpression of *β*-catenin/Tcf targets such as c-Myc and c-Jun (Blum *et al*., 2001), and the elevated expression of Bcl-2 protein with loss of Bax (Hayashi *et al*., 1996). In the present investigation of PhIP-induced colon tumors, a striking concordance was found between the presence of *β*-catenin mutations and increased *Bcl-2*/*Bax* mRNA expression (Table 1), suggesting that *β*-catenin/Tcf might activate the rat *Bcl-2* gene (and/or inhibit Bax) at the transcriptional level.

The *Bcl-2* proto-oncogene is frequently expressed in human cancers, and Bcl-2 is regulated both transcriptionally and post-transcriptionally (Bedi *et al*., 1995; Harigai *et al*., 1996). We cloned and characterized the rat *Bcl-2* promoter for the first time and found a similar genomic organization as the human counterpart, *BCL-2* (Seto *et al*., 1988; Harigai *et al*., 1996), with a core promoter that shares 85% identity between the two species. We also identified transcription factors bound to the rat *Bcl-2* promoter, and excluded several putative Tcf sites on the basis of the results from protein/DNA arrays (Figure 2) and mobility-shift assays (data not shown). Thus, the rat *Bcl-2* gene does not appear to be a direct *β*-catenin/Tcf target.

Various transcription factors have been implicated as regulators of Bcl-2 (Wilson *et al*., 1996; Salomoni *et al*., 1997; Smith *et al*., 1998; Mayo *et al*., 1999; Pugazhenthi *et al*., 1999; Romero *et al*., 1999; Tamatani *et al*., 1999; Grossmann *et al*., 2000). Human *BCL-2* contains an authentic E2F response element (Gomez-Manzano *et al*., 2001) and was identified as an E2F1 target gene by cDNA microarray analysis (Muller *et al*., 2001). We report here, for the first time, that E2F1 also directly regulates the rat *Bcl-2* gene, with strong signals in protein/DNA arrays (Figure 2) and loss of promoter activity upon deletion of the E2F1 sites (Figure 3).

Rat *Bcl-2* promoter activity was increased by LiCl treatment and by exogenous wild-type and mutant *β*-catenins. Lithium has been shown to stabilize *β*-catenin via inhibition of GSK-3*β* (Behrens, 2005), and increased Bcl-2 levels in rat frontal cortex, hippocampus and striatum, as well as in cultured retinal ganglion cells (Manji *et al*., 2000; Huang *et al*., 2003). Of particular interest, however, oncogenic mutants of *β*-catenin from PhIP-induced colon tumors strongly activated *Bcl-2* promoter activity and Bcl-2 protein expression, supporting a link between increased *β*-catenin and Bcl-2 (Figure 4c).

c-*Myc* is a well-known *β*-catenin/Tcf target (He *et al*., 1998), and is strongly overexpressed in rat colon tumors both at the mRNA and protein level (Blum *et al*., 2001; Fujiwara *et al*., 2004). Interestingly, a recent report indicated that c-Myc-regulated microRNAs modulate E2F1 expression (O'Donnell *et al*., 2005). In our experiments, knockdown of c-Myc by siRNA blocked the induction of E2F1 and Bcl-2 by *β*-catenin, as well as inhibiting rat *Bcl-2* promoter activity (Figure 5a), and knockdown of E2F1 by siRNA also attenuated *Bcl-2* reporter activity and Bcl-2 protein expression (Figure 5b). We conclude that an indirect pathway exists between

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β-catenin and Bcl-2 in PhIP-induced colon tumors, in which mutations in *β*-catenin activate *β*-catenin/Tcf signaling, increase c-Myc, elevate E2F1 expression and enhance Bcl-2 expression. Further studies of this pathway are warranted, including the possible contribution of miroRNAs in PhIP-induced colon tumors and early lesions such as colonic aberrant crypts and dysplastic foci (Ochiai *et al*., 2003).

Materials and methods

Source of colon tumors

Colon tumors were from a study in which male F344 rats were treated with PhIP, as reported previously (Dashwood *et al*., 1998). At necropsy, one portion of each tumor was taken for histopathology, and other portions were frozen in liquid nitrogen for molecular analyses.

Competitive RT-PCR

Total RNA was isolated from colon tumors and adjacent normal looking tissue using RNeasy RNA isolation kit (Qiagen, Valencia, CA, USA). cDNAs were amplified with primers specific for *Bcl-2* or *Bax* in the presence of serial dilutions of competitor DNA (Clontech, Palo Alto, CA, USA). Parallel reactions were run with primers and competitor DNA for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). PCR products were separated on 2% agarose gels, visualized by ethidium bromide staining and quantified on an AlphaImager 2200 (AlphaInnotech, San Leandro, CA, USA). *Bcl-2* or *Bax* levels, normalized relative to *GAPDH*, were expressed for tumor versus normal looking tissue.

Mutation screening

PhIP-induced colon tumors and adjacent normal looking tissue were subjected to DNA isolation and PCR-based single strand conformation polymorphism (PCR-SSCP) analyses, using the experimental conditions reported previously (Dashwood *et al*., 1998).

Cloning of the 5′-flanking region of *Bcl-2*

The 5′-flanking region of the rat *Bcl-2* gene was amplified using the Rat GenomeWalker kit (Clontech). The primary PCR was performed with Adapter Primer 1, supplied with the kit and gene-specific primer rBcl2P-GSP1 (5′-TGCATTCT TG GATGAAGGGGTGTCTT-3′). Subsequently, secondary PCR was performed with Adapter Primer 2 (supplied with the kit) and a nested gene-specific primer rBcl2P-GSP2 (5′-TCCCCCTTGGCATGAGATGCAGGAAAT-3′). The primary PCR was performed for 35 cycles at 98°C for 20 s and 65°C for 4 min, with an additional 10 min extension at 72°C after the final cycle. The nested PCR was run for 30 cycles, denaturing for 20 s at 98°C, annealing for 5 min at 68°C, and ending with a final extension for 10 min at 72°C. PCR products were subcloned into pGEM-T (Promega, Madison, WI, USA) and subjected to DNA sequencing in both directions on an ABI Prizm model 377 sequencer (Applied Biosystems, Bedford, MA, USA). Based on the new sequence, primers rBcl2P-GSP3 (5′-GGGAACGGGGACCAGAATCCTCTTCT-3′) and rBcl2P-GSP4 (5′-TTAAACTCCGAAGGGCCAATGCG TTTTC-3′) were used to obtain additional flanking sequence of *Bcl-2*.

5′-Rapid amplification of cDNA ends (5′-RACE)

The transcription start site was identified using 5′-RACE System Version 2.0 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. First strand cDNA was synthesized from 100 ng rat liver poly(A)$^+$RNA using an antisense gene-specific primer RA-b2-GSP1 (5′-CCTCT GTGACAGCTTAT-3′). The cDNA was purified and an oligo-dC tail was added to the 3′end of the cDNA using terminal transferase TdT. Homopolymeric C tailed

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cDNA was then amplified by PCR using Abridged Anchor Primer (supplied with the kit) and a nested gene-specific primer RAb2- GSP2 (5′-CGGTTATCATACCCTGTTCTCCCGGCTT- 3′). PCR tubes were transferred from ice to a thermal cycler pre-equilibrated to 94°C, and after 2–3 min 35 cycles were performed of 30 s/94°C, 30 s/55°C and 60 s/72°C, with final extension at 72°C for 10 min. The PCR product was further amplified using nested primers AUAP (supplied with the kit) and RA-b2-GSP3 (5′-GAAGCTGCAGGTACCAATAGCA CTT-3′), and cycling parameters identical to the first round. 5′-RACE products were purified using Wizard PCR Preps DNA Purification System (Promega) and sequenced using primer RA-b2-GSP3.

Construction of Plasmids

Progressive deletion constructs of the rat *Bcl-2* promoter, including Bcl2-P1 (−1945 to −906) and Bcl2-P2 (−905 to −1), were engineered by cloning PCR fragments between *Kpn*I and *Xho*I sites of the reporter luciferase vector pGL3Basic (Promega); primer sequences are available upon request. Plasmids containing one or two mutant E2F1-binding sites were generated from pGL3-Bcl2-P1 by site-directed mutagenesis using the QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). To generate pcDNA3.1-E2F1, an E2F1 expression construct, full-length human E2F1 cDNA was amplified by RT-PCR. The PCR products were cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) between *Bam*HI and *Eco*RV sites. According to the same methodology, plasmids expressing AP2*α*, c-Rel, c-Myb, GATA4, MEF2, NF-1, p53, PARP and STAT4 were constructed by subcloning the corresponding full-length cDNA into pcDNA3.1(+). The wild-type *β*-catenin cDNA construct pcDNAI/*β*-catenin was kindly provided by Hans Clevers and Marc van de Wetering. Oncogenic *β*-catenin mutants, generated by fragment switching, were as described before (Dashwood *et al*., 2002; Al-Fageeh *et al*. 2004; Dashwood *et al*., 2005). All constructs were confirmed by sequencing in both directions.

c-Myc and E2F1 knockdown by siRNA

Inhibition of c-Myc expression in HEK293 cells was performed using SureSilencing Human MYC siRNA and Antibody Kit (SuperArray Biosciences, Frederick, MO, USA). Cells were transfected with MYC-specific siRNA population using Lipofectamine2000 (Invitrogen) as recommended by the manufacturer. Non-specific siRNA was used as negative control. In subsequent experiments, E2F1 was knocked down using ON-TARGETplus SMARTpool human E2F1 siRNA from Dharmacon (Chicago, IL, USA).

Cell culture and transient transfection experiments

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum (Invitrogen). Rat kidney epithelial (RK3E) cells were grown in DMEM supplemented with 10% bovine fetal serum (Invitrogen). Cultures were maintained at 37°C in a humidified 5% CO_2-containing atmosphere. Transfection was performed using TransFast (Promega) or FuGENE 6 (Roche, Palo Alto, CA, USA) following manufacturer's instructions, and cells were harvested 48 h after transfection. To inhibit GSK-3*β* and induce endogenous *β*-catenin, 30mM LiCl was added to culture medium; 30mM NaCl was used as control.

β-Galactosidase (β-Gal) and luciferase assays

β-Galactosidase and luciferase assays were performed as reported previously (Li *et al*., 2004).

Isolation of transcription factors bound to the rat *Bcl-2* promoter

Transcription factors bound to the 1040 bp *Bcl-2* promoter 1 (−1945 to −906) were purified by DNA pull-down assays according to the procedure described previously (Li *et al*., 2004).

Bcl-2 promoter fragments were end labeled with biotin using Bio-16-dUTP (Enzo Life Sciences, Farmingdale, NY, USA) and the Klenow fragment of DNA polymerase I (Fermentas Inc., Hanover, MD, USA). Proteins were eluted on ice in 50 μl of TGED buffer containing 2M NaCl.

Protein/DNA array analyses

After DNA pull-down assays, TranSignal Protein/DNA Arrays I and II (Panomics, Redwood, CA, USA) were used to identify the transcription factors associated with the rat *Bcl-2* promoter, as described before for the β-catenin gene *Ctnnb1* (Li *et al*., 2004).

Western blotting

Whole cell lysates were prepared using Reporter Lysis buffer (Promega) and the protein concentration was determined as reported (Li and Dashwood, 2004; Li *et al*., 2004). Proteins were separated on 4–12% bis-tris gels (Novex, Invitrogen) and transferred to nitrocellulose membranes (Invitrogen), and after incubation with primary antibody followed by secondary antibody conjugated to horseradish peroxidase, detection was by Western Lighting Chemiluminescence Reagents Plus (Perkin Elmer Life Science, Boston, MA, USA).

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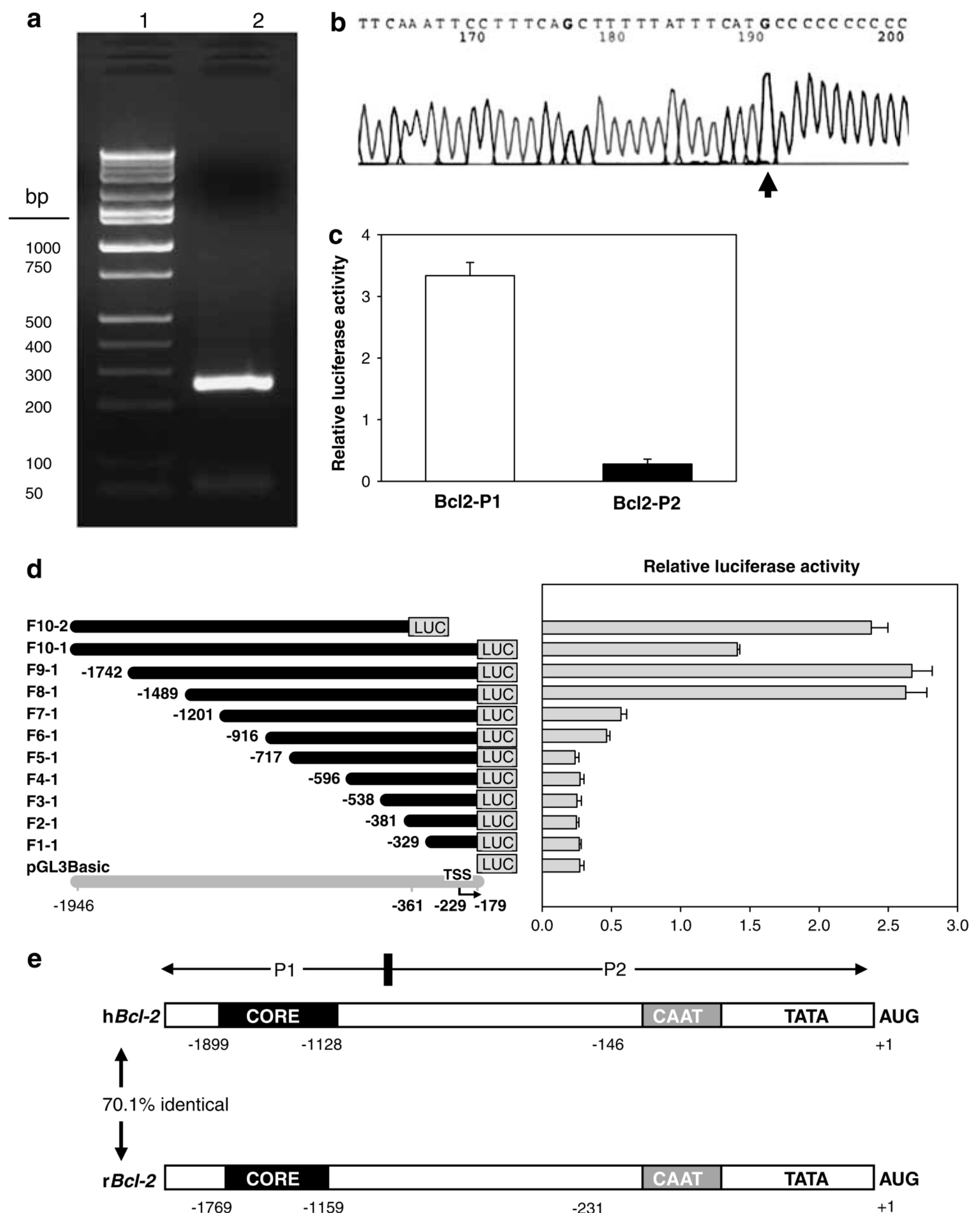

Figure 1.

Characterization of the rat Bcl-2 promoter. (**a**) Product from 5′-rapid amplification of cDNA ends (5′-RACE) on a 2% agarose gel stained with ethidium bromide. In 5′-RACE, fresh poly $(A)^{+}$RNA (Ambion) was used to ensure a high percentage of full-length transcripts, which consistently generated a *single* 260 bp PCR product (lane 2; lane 1=marker ladder). Degradation of full-length poly$(A)^{+}$RNAs was discounted based on the lack of shorter or longer 5′-RACE products in repeat experiments, and DNA sequencing (GenBank accession no. AF531426) indicated a common 5′ end with the major TSS assigned to a C nucleotide 231 bp upstream of the translation start site (**b**), consistent with the human *BCL-2* counterpart. (**c**) *Bcl-2* promoter-luciferase reporter construct containing promoter region P1 had >10-fold higher activity than region P2 when transfected into HEK293 cells and luciferase activities were measured in whole cell lysates 48 h post-transfection. (**d**) Deletion analysis. Left panel: schematic view of *Bcl-2* promoter-luciferase reporter constructs; gray line, position in genomic

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DNA sequence; bent arrow, transcription start site (TSS); black lines, extent of genomic DNA attached to the luciferase (LUC) gene. Promoter fragments were designated as F1-1 through F10-2, as shown at left. Right panel: *Bcl-2* promoter activity in HEK293 cells. Transcriptional activity was evaluated by transient transfection, using the constructs shown in the left panel. Cells were extracted using 1×Reporter lysis buffer, 48 h post-transfection. Values represent the mean±s.d. of three independent transfections for each construct, normalized for transfection efficiency using pSV-*β*-Gal, as reported before (Li *et al*., 2004). (**e**) Genomic organization of human *BCL-2* (h*BCL-2*) and rat *Bcl-2* (r*Bcl-2*) promoters, with numbering relative to the translation start codon (AUG). CORE, core promoter region.

a Bcl-2 P1 (Array I)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
AP1	AP1	AP2	AP2			ARE	ARE			CBF	CBF	CDP	CDP	c-Myb	c-Myb			A
																		B
CREB	CREB	E2F1	E2F1	EGR	EGR											AP2	AP2	C
		E2F1	E2F1															D
GATA	GATA	GRE	GRE					MEF1	MEF1					NF-1	NF-1			E
								MEF1	MEF1									F
				NFE2	NFE2	NFkB	NFkB			p53	p53	Pax5	Pax5	Pbx1	Pbx1			G
						NFkB	NFkB											H
						RAR	RAR			SIE	SIE			Smad	Smad			I
																		J
								Stat4	Stat4									K
																		L
				USF1	USF1													M
																		N

b Bcl-2 P1 (Array II)

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
																A
CEF1	CEF1	CEF2	CEF2	CETF	CETF											B
														EIK1	EIK1	C
																D
										GATA3		GATA4				E
																F
																G
ISRE2	ISRE2									MEF2	MEF2					H
						NF-Y	NF-Y									I
				PARP	PARP			Pax3	Pax3	Pax4	Pax4					J
		TCF	TCF	REB1R	REB1R	RREB2	RREB2	RSRFC4	RSRFC4			Skn	Skn			K
				XP1B	XP1B							ZIC	ZIC			L

Figure 2.

Transcription factors bound to the rat *Bcl-2* promoter. Nuclear proteins bound to a 1040 bp (−1945 to −906) fragment of r*Bcl-2* promoter region P1 were pulled down from nuclear extracts of HEK293 cells and transcription factors were identified using TranSignal protein/DNA Array I (**a**) and Array II (**b**) (Panomics), as reported (Li *et al*., 2004). Top panels, hybridization signals; bottom panels, transcription factor identities. Box, Tcf signal; dotted box, E2F1 signal.

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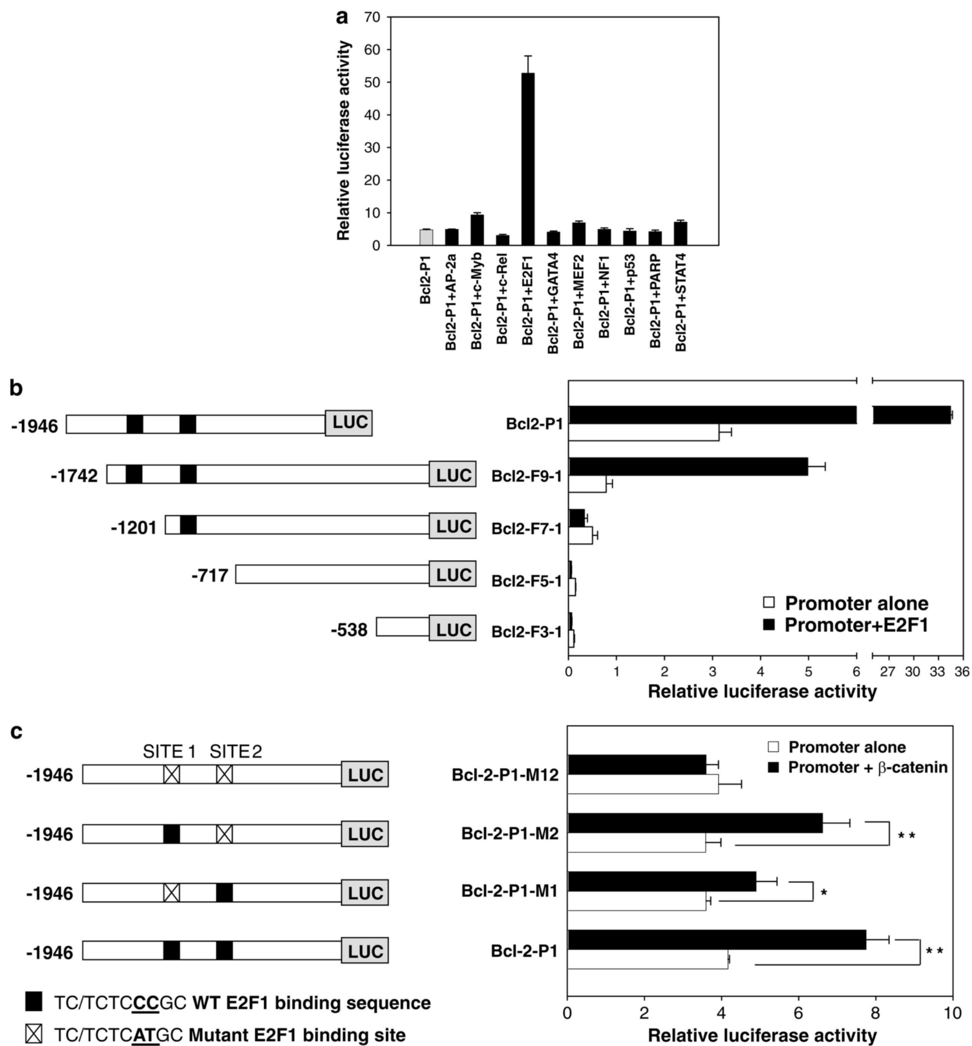

Figure 3.

Confirmation of E2F1 sites in the rat *Bcl-2* promoter. (**a**) HEK293 cells were transfected with *Bcl-2*-P1 plus constructs expressing transcription factors identified in protein-DNA arrays (Figure 2). pSV-*β*-Gal was used as internal control. Cell lysates were analyzed for luciferase and *β*-Gal activities 48 h post-transfection. Luciferase activity was normalized to *β*-Gal to obtain the relative luciferase activity. E2F-1 binding sites were confirmed using (**b**) deletion analyses and (**c**) site-directed mutagenesis. Left, diagram of *Bcl-2* promoter-luciferase constructs. Right, *Bcl-2* promoter activities in the presence and absence of exogenous (**b**) E2F1 or (**c**) *β*-catenin. Data, mean±s.d., n=3. WT, wild-type. *P<0.05; **P<0.01.

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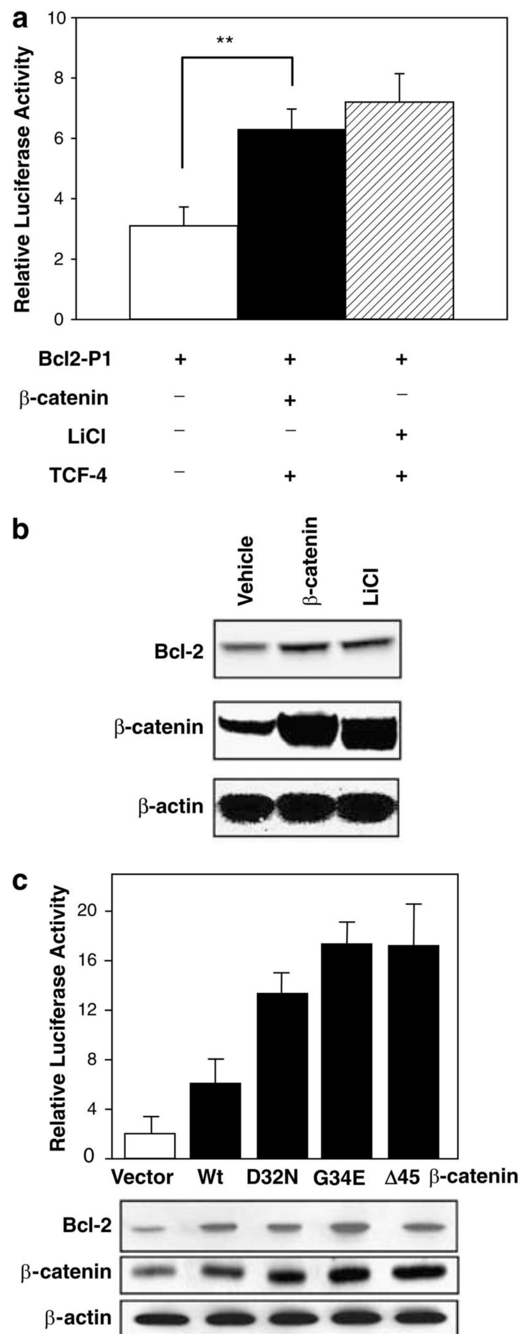

Figure 4.

Regulation of *Bcl-2* promoter activity by *β*-catenin. (**a**) Induction of *Bcl-2* promoter activity by forced expression of *β*-catenin. HEK293 cells were transfected with *Bcl-2*-P1 promoter-luciferase construct alone, or *Bcl-2*-P1 plus a construct that overexpressed wild-type *β*-catenin; pSV-*β*-Gal was used as internal control. Alternatively, endogenous *β*-catenin was overexpressed with 30mM LiCl. (**b**) Immunoblot of whole cell lysates showing increased Bcl-2 and *β*-catenin following exogenous *β*-catenin or LiCl treatment. (**c**) Induction of *Bcl-2* promoter–reporter activity by wild-type (Wt) *β*-catenin and *β*-catenin mutants D32N, G34E and Δ45, with concomitant changes in *β*-catenin and Bcl-2 protein expression in whole cell lysates.

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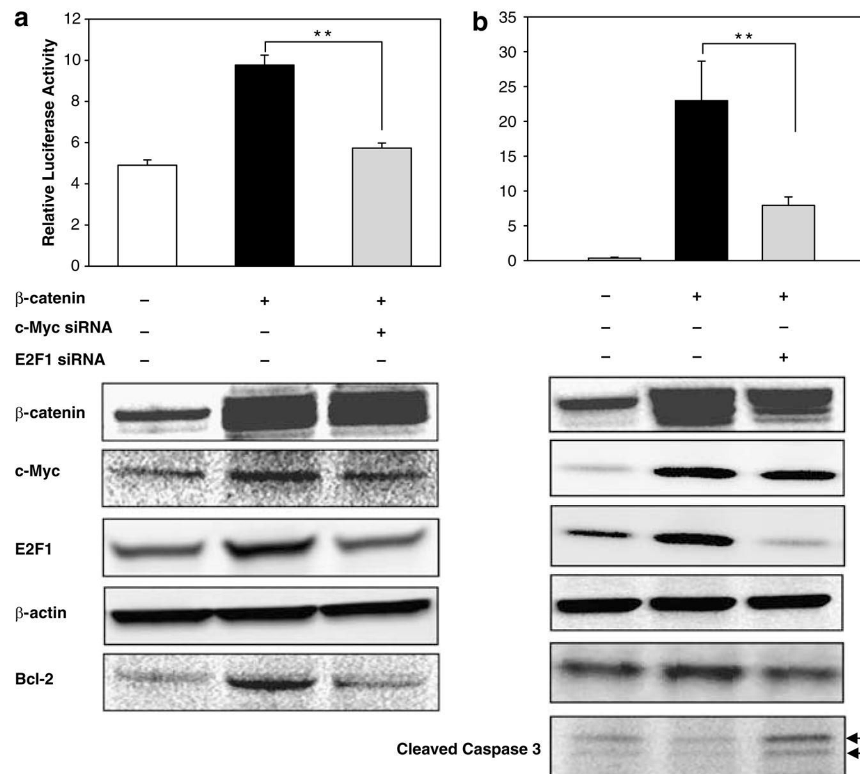

Figure 5.
Knockdown of c-Myc or E2F1 attenuates *β*-catenin-dependent induction of *Bcl-2* promoter activity and Bcl-2 protein expression. HEK293 cells were transfected with *Bcl-2*-P1, in the presence and absence of exogenous *β*-catenin, plus c-Myc siRNA or E2F1 siRNA. Luciferase and *β*-Gal activities were assayed 48 h post-transfection. Luciferase activity was normalized to *β*-Gal activity to obtain the Relative Luciferase Activity. Data, mean±s.d., n=3; **P<0.01. Corresponding whole cell lysates were immunoblotted for *β*-catenin, c-Myc, E2F1, *β*-actin, Bcl-2 and cleaved caspase 3, as indicated. Arrows, 19-kDa and 17-kDa bands indicative of cleaved (active) caspase 3.

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Table 1

Bcl-2/Bax expression and *β*-catenin mutation status in PhIP-induced colon tumors

Tumor ID #	Relative *Bcl-2* mRNA expression[a]	Relative *Bax* mRNA expression[a]	*Bcl-2*/*Bax* ratio	*Ctnnb1* mutation status[b]
98-01-18	4.0	0.5	8	GGA→GAA (G34E)
98-02-16	0.5	1.0	0.5	None detected
98-02-18	4.0	0.5	8	GGA→GAA (G34E)
98-02-20	4.0	0.5	8	GGA→GTA (G34 V)
98-02-22	1.0	1.0	1	None detected
98-02-23	1.0	1.0	1	None detected

Abbreviations: PhIP, 2-amino-1-methyl-6-phenylimidazo(4,5-*b*)pyridine.

[a]Tumor versus adjacent normal-looking tissue.

[b]detected by PCRbased single strand conformation polymorphism analysis and direct sequencing (Dashwood *et al*., 1998), data not presented. *Ctnnb1*, gene designation for rat *β*-catenin.