Identification of the Polycomb Group Protein SU(Z)12 as a Potential Molecular Target for Human Cancer Therapy[1]

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Abstract

We have previously identified *SU(Z)12* as an E2F target gene. Because many E2F target genes encode proteins that are critical for the control of cell proliferation, we have further characterized the regulation and expression of *SU(Z)12*. To understand the molecular mechanisms responsible for expression of *SU(Z)12* mRNA, we have analyzed the promoter region. We found that the *SU(Z)12* gene is controlled by dual promoters, one of which functions bidirectionally. In addition to the E2F binding site, we have identified two binding sites for T cell factor (TCF)/β-catenin complexes. Using gel mobility shift assays, we demonstrated that both TCF sites can be bound by TCF4. TCF/β-catenin complexes have been shown to be a critical regulator of gene expression in tumors of the colon, breast, and liver. Accordingly, we have used chromatin immunoprecipitation assays to confirm that TCF4/β-catenin complexes are bound to the *SU(Z)12* promoter in colon cancer cells but not in HeLa cells. We next adapted the chromatin immunoprecipitation assay for use with primary colon tumor samples, and, using matched pairs of normal and tumor tissue obtained from several different colon cancer patients, we demonstrate that levels of β-catenin bound to the *SU(Z)12* promoter are increased in colon tumors. Finally, we show that the *SU(Z)12* mRNA is up-regulated in a number of different human tumors, including tumors of the colon, breast, and liver. Recent studies have found that SU(Z)12 is a component of the *Drosophila* ESC-E(Z) and the human EED-EZH2 Polycomb chromatin remodeling complexes. Therefore, we suggest that SU(Z)12, which may modulate the tumor phenotype by changing gene expression profiles, may be a logical target for the design of a new antitumor agent.

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Introduction

We had previously identified the human *SU(Z)12* [also known as KIAA0160, *JJAZ1*, and *ChET9* (1–4)] promoter as one of several chromatin fragments that were isolated by virtue of their *in vivo* interaction with E2F transcription factors (4). E2Fs regulate the expression of genes involved in nucleotide metabolism, DNA replication, cell cycle control, apoptosis, DNA repair, and DNA replication (5–8). Many of these E2F target promoters have been shown to be responsive to changes in cell growth conditions. For example, E2F target genes often show increased expression in highly proliferating normal tissues and in certain tumor types (9). In fact, an E2F target promoter has been used to achieve selective killing of tumor cells (10). Much of the proliferation-specific expression of E2F target genes is attributable to their regulation by the Rb[3] tumor suppressor family of proteins, which includes Rb, p107, and p130. Interaction of Rb, p107, or p130 with the E2F factors results in transcriptional repression of target genes. However, in many tumors this interaction is abolished by mutation of the Rb family members or increased expression or activity of cyclin/cdk complexes, which function to phosphorylate Rb and break up the Rb/E2F interaction (11). The loss of Rb-mediated repression allows E2F to activate its target genes to high levels in tumor cells. Tumor-specific expression of E2F target genes may also be achieved via cooperation of E2F family members with nuclear oncogenes. For example, E2F cooperates with Myc to induce cell transformation (9) and, at least in certain cases, it has been suggested that both transcription factors regulate the same promoter (12–14). We have also noted a correlation between E2F target promoters and β-catenin target promoters. For example, the *Myc* and *cyclin D1* promoters, both of which are regulated by β-catenin (15, 16), are also regulated by the E2F family (17, 18). Also, we have shown that the *Myc*, *cyclin D1*, *jun,* and *PPARδ* promoters are all bound by both β-catenin and E2F4 *in vivo*.[4] Although no direct interaction between E2F4 and β-catenin has been reported, it is possible that the two factors cooperate to activate transcription via a mechanism such as transcription factor synergy (19).

The observed coincidence of E2F and β-catenin target promoters suggests that the expression of certain E2F target genes may be altered in cancers caused by the deregulation of β-catenin activity, such as colon cancer. Colorectal cancer is the third leading cause of cancer death in the United States. The majority of colorectal tumors contain mutations in the *APC* tumor suppressor gene (20). Normal APC protein

[3] The abbreviations used are: Rb, retinoblastoma; APC, adenomatous polyposis coli; RT-PCR, reverse transcription-PCR; PcG, Polycomb group; ESC, extra sex comb; ChIP, chromatin immunoprecipitation; EST, expressed sequence tag; DEN, diethylnitrosamine; TCF, T cell factor; PRC2, polycomb repressive complex 2.

[4] A. Kirmizis, unpublished observations.

has been shown to bind to and down-regulate the level of β-catenin, a component of cell adhesion complexes. Most APC mutations found in colorectal tumors result in truncated APC proteins that lack the region required for down-regulation of β-catenin. Thus, in colon tumors, β-catenin accumulates to high levels and translocates to the nucleus. Yeast two hybrid screens identified β-catenin as an interaction partner of the TCF/Lef family of transcription factors. Overexpression of β-catenin in tissue culture cells can cause transcriptional up-regulation of certain promoters that contain TCF binding sites (16) and a direct fusion of β-catenin to the Lef-1 DNA binding domain can activate transcription (21). Therefore, based on the fact that loss of APC leads to increased β-catenin and that increased β-catenin can cause transcriptional activation, it has been postulated that the abnormal expression of genes regulated by a TCF/β-catenin complex is a critical determinant of the neoplastic phenotype of colon cancer cells (22). To date, several putative β-catenin target genes have been identified by comparing gene expression profiles in populations of cells grown in tissue culture. These genes include *fra-1*, *c-jun*, *c-Myc*, *matrilysin*, *cyclin D1*, *PGHS-2*, and *PPARδ* (15, 16, 23–28). Although the identification of these β-catenin target genes has provided much new insight into colon cancer, it is likely that additional genes important in neoplastic transformation of colon cells remain to be discovered.

We have now expanded our analysis of the *SU(Z)12* gene and have found that the *SU(Z)12* promoter is bound by both E2F4 and TCF/β-catenin complexes. We also show that the levels of β-catenin recruited to the *SU(Z)12* promoter increase in colon tumors, as compared with normal colon tissue from the same patient, and that this increased binding of β-catenin correlates with increased levels of *SU(Z)12* mRNA.

Materials and Methods

RT-PCR Analysis. For each RT-PCR reaction, 100 ng of mouse liver RNA, prepared as described previously (29), was analyzed at a hybridization temperature of 63° for 32 cycles. Human colon and breast RNA, prepared as described previously (30), was a gift from Jeff Ross, who obtained the tissue from the Cooperative Human Tissue Network (which is funded by National Cancer Institute). We note that the normal samples used for these experiments and for the ChIP experiments described below may have slight contamination with the underlying stromal cells. Fortunately, the three-dimensional architecture of the colon tumors allows the tumor samples to be removed from the colon without encroaching on the adjacent stromal cells. For each RT-PCR reaction using human RNA, performed as described previously (31), 100 ng of RNA was analyzed at a hybridization temperature of 59° for 28 cycles. Primers used in the reactions are listed in Table 1; all of the primers used in RT-PCR, PCR, cloning, and gel shifts were obtained from the University of Wisconsin Biotechnology Center. All of the work using human tissues for either RNA analysis or ChIP experiments was performed under guidelines of the NIH and the University of Wisconsin human subjects Institutional Review Board.

Primer Extensions. RNA was prepared from HeLa cells as described previously (32). When using primer A, 10 μg of HeLa mRNA was included in the reaction; when using primer B, 5 μg of HeLa mRNA was included. The sequences of primer A and primer B are listed in Table 1. Primer extensions were performed as described previously (33, 34).

ChIP. ChIPs using cultured cells were performed as described previously (4). For the analysis of colon tissue, the ChIP protocol required several modifications.[5] Briefly, the protocol required mincing the tissue in PBS, cross-linking in formaldehyde for a longer time than used for tissue culture cells, and then processing with a Medi-machine to achieve single cells. Antibodies used were to RNA polymerase II (Santa Cruz Biotechnology; sc-899), E2F4 (Santa Cruz Biotechnology; sc-866X), TCF4 (Santa Cruz Biotechnology; 8631X), TCF3/4 (Upstate Biotechnology; 05-512), and β-catenin (Transduction Laboratories; C19220). The sequences of the primers used in the ChIP assays are listed in Table 1.

Plasmid Constructs. Promoter reporter constructs were prepared by PCR using primers spanning the indicated regions. The sequence of the primers used to clone the promoter constructs is listed in Table 1. Each primer also contained a *Hin*dIII site at the 5′ end to facilitate cloning of the PCR fragments into the *Hin*dIII site in pGL2 basic (Promega Inc., Madison, WI). Genomic human DNA was used as a template for the PCR reactions. Each fragment was cloned in both orientations to allow an analysis of bidirectional promoter activity.

Transient Transfections. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's media (Life Biotechnology, Inc., Grand Island, NY), supplemented with 5% bovine calf serum (Hyclone, Logan, UT) and 1% penicillin/streptomycin, and incubated at 37°C in a humidified 5% CO2 incubator. Transient transfections using the calcium phosphate method were performed as described previously (32). For the analysis of promoter activity, 2 μg of reporter constructs plus 13 μg of sonicated salmon sperm DNA was transfected into proliferating cells, which were harvested 48 h after transfection; experiments were performed in triplicate, and each transfection experiment was carried out in duplicate plates.

Electromobility Shift Assays. *In vitro* TCF/β-catenin DNA-binding activity was assayed using electromobility shift assay competition experiments. Approximately 6 μg of HT29 whole cell extract, prepared as described previously (35), was incubated with 0.3 μg of sonicated herring sperm DNA and 2 μl of binding buffer [50 mM HEPES (pH 7.4), 300 mM KCl, 5 mM EDTA, 5 mM DTT, 11.5% Ficoll] in a total volume of 18 μl for 15 min at room temperature. About 40,000 cpm of the [γ-^{32}P]ATP-labeled optimal TCF (Opt-TCF) oligonucleotide (36) was then added to the binding reactions, and the incubation continued for an additional 20 min. The double-stranded oligonucleotides used as competitors were either the unlabeled probe or oligonucleotides based on sequences from the *SU(Z)12* promoter (TBS1, TBS1mt, TBS2,

[5] The several modifications required by ChIP protocol are described in detail at http://mcardle.oncology.wisc.edu/farnham/.

Table 1 Primer sequences

All of the primers are listed in the 5′ to 3′ orientation. The consensus TCF site (a/t,a/t,caaag) is underlined in each oligonucleotide used in the gel shift experiments.

Assay	Primer sequence
RT-PCR (human)	
Left	cttacatgtctcatcgaaactcc
Right	ggctggaagctcttcattgaca
RT-PCR (mouse)	
Left	gccgaaaatggagcacgtccaggc
Right	cacaagcaggacttccagggtaac
Primer extension	
A	ccaaccgctcgccccagcaggctcc
B	ctgtgttggtttctcaaaggcctgg
Cloning	
Upstream promoter	
Left	tacgataagcttggtggcgagcgcctgtaatc
Right	tacgataagctttgtctcctcagagccccgct
Downstream promoter	
Left	tacgataagctttcggctagccggagcctgct
Right	tacgataagcttcttcttcaccggtaacgccg
ChIP:	
SU (Z) 12	
Left	tcaccctaccctggcctcgct
Right	tcgctaaaccgctcgctgggt
Myc	
Left	acagacgcctcccgcacggg
Right	ccacaccgagaacgcactgc
Cyclin D1	
Left	cggactacaggggagttttgttg
Right	tccagcatccaggtggcgacgat
H2A	
Left	ggagcagacttcgctggctctg
Right	gcgcaactcactacgagcaacc
B-Myb	
Left	ggtgttcgctatgtgggata
Right	ctcctcgctcgcaggaactg
Gel shift	
Opt-TCF	ggtaagatcaaaggg
Proximal	
TBS1	agcttcgcgaattcagttcaaagaggcggccgggca
TBS1mt	agcttcgcgaattcaggccagagaggcggccgggca
Distal	
TBS2	agctttgtgtctatcgttcaaagcaagacctggcca
TBS2mt	agctttgtgtctatcggccagagcaagacctggcca

TBS2mt) and were included in the first incubation at a 10-fold molar excess to the labeled probe. The sequences of the oligonucleotides used in the binding reactions are listed in Table 1. For supershifts, 1.5 μl of mouse anti-β-catenin monoclonal 15B8 ascites fluid (Abcam Ltd.; ab6301–100), 1 μg of TCF4 antibody (Santa Cruz Biotechnology; 8631X), or 1 μg of HNF3γ antibody (Santa Cruz Biotechnology; 5360X) were included in the first incubation. The reactions were electrophoresed for ~3 h on a 4.5% polyacrylamide gel that had been pre-electrophoresed for 30 min.

Results

Dual Promoters Drive Expression of *SU(Z)12* mRNA. A full-length cDNA corresponding to the human *SU(Z)12* mRNA has previously been deposited in GenBank (accession no. D63881; Ref. 1). Inspection of the collection of ESTs and cDNAs that map to the location of the *SU(Z)12* gene, as determined using the University of California-Santa Cruz human genome database,[6] indicated that several other groups have cloned mRNAs that have 5′ ends mapping near the 5′ end of the original cDNA. However, two ESTs extend slightly upstream of the cloned cDNA and several end between 300 and 400 nucleotides downstream of the 5′ end of the cloned cDNA. Interestingly, the shorter human ESTs have 5′ ends mapping near the 5′ end of the corresponding mouse cDNA (GenBank accession no. BF658962). These results suggested that there may be two different transcription start sites, one located near the 5′ end of the human cDNA clone and one located near the 5′ end of the mouse cDNA clone. We noted that consensus Sp1 and E2F sites can be found at distances appropriate to serve as promoter elements for mRNA coming from both putative start sites (see Fig. 1). Therefore, as a first step in characterizing the transcriptional

[6] Internet address: http://genome.cse.ucsc.edu/.

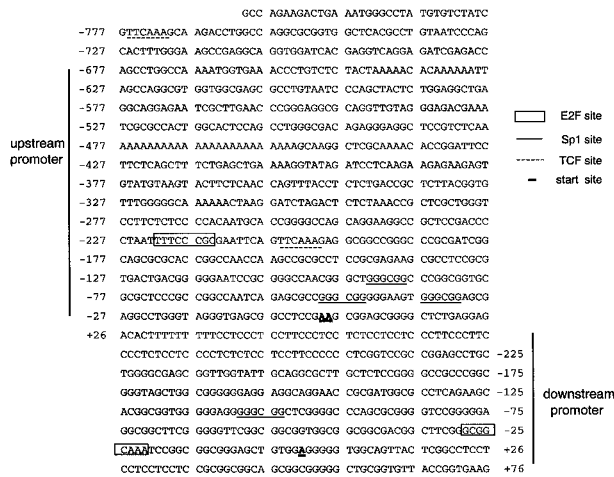

Fig. 1. Sequence of the *SU(Z)12* promoter. The genomic sequence representing the *SU(Z)12* promoter region is shown. Indicated are the consensus E2F, Sp1, and TCF sites. *Bold type* and *underlined*, the positions of the transcription initiation sites, as determined by primer extension analysis. *The numbers to the left of the sequence* are calculated relative to the upstream start site; *the numbers to the right of the promoter* are calculated relative to the downstream start site.

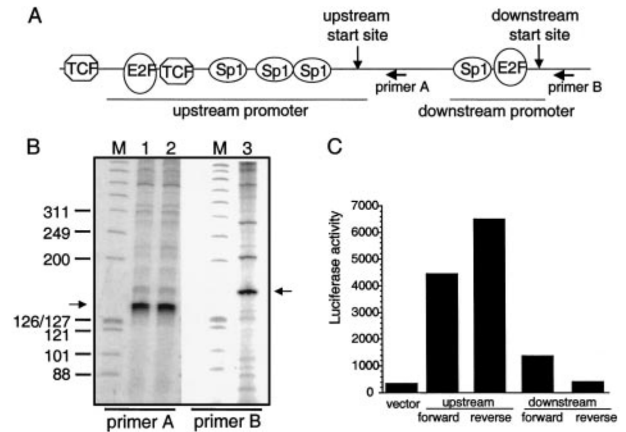

Fig. 2. Dual promoters drive expression of *SU(Z)12* mRNA. *A*, schematic of the human *SU(Z)12* promoter. Shown are the relative locations of the consensus Sp1, E2F, and TCF sites, the location of the primers used for primer extension analyses, the approximate location of the upstream and downstream transcription initiation sites, and the fragments used in panel *C* for promoter analyses. *B*, primer extension analysis was performed using HeLa cell RNA and primer A and primer B. *Lanes 1* and *2*, two different reactions using the same primer and RNA samples. *Lanes M*, DNA markers (sizes shown *to the left of the panel*). *Arrow on the left*, the upstream transcription initiation site; *arrow on the right*, downstream transcription initiation site. The gel representing the *right panel*, containing samples analyzed by *primer B*, was exposed longer than the left panel so that an easily visible signal could be obtained. *C*, the *upstream* and *downstream* promoter fragments were cloned upstream of the luciferase cDNA in both orientations. The plasmid constructs were transiently transfected into NIH 3T3 cells, and luciferase activity was monitored as described previously (32). The vector (pGL2) sample was obtained by transfection of the luciferase reporter plasmid lacking an inserted promoter fragment. In each case, the *forward* orientation indicates that the fragment is placed in the orientation found in the genome, relative to *SU(Z)12* mRNA.

regulation of the *SU(Z)12* gene, we used primer extension analysis to map the 5′ end of the *SU(Z)12* mRNA from human cells.

To map the 5′ end of the human *SU(Z)12* mRNA, two different primers were prepared; mRNAs beginning in the region corresponding to the 5′ end of the cloned human cDNA should be easily mapped using primer A, and mRNAs beginning in the region corresponding to the 5′ end of the cloned mouse cDNA should be easily mapped using primer B. Using primer A, we found strong signals that corresponded to a position ~11 bp upstream of the cloned human cDNA (Fig. 2*B*). Interestingly, this transcription start site maps to the same position as one of the longest ESTs (GenBank accession no. BG655887). Using primer B, we detected a slightly weaker signal (Fig. 2*B*) that maps to a position near the 5′ end of the shorter ESTs (*e.g.,* GenBank accession no. AA356424). To ensure that signals from primer B were not weaker because of problems with labeling the primer and/or inefficient hybridization of that particular primer, we prepared another primer complementary to the sequences adjacent to primer B; again, weaker signals were obtained using this primer (data not shown). Therefore, it appears as though human *SU(Z)12* mRNA is produced from two start sites, with the upstream region corresponding to the majority of the mRNA.

The results of the primer extension analyses, which indicate that transcription initiates just downstream of both sets of E2F and Sp1 sites, suggest that perhaps two different promoter regions are used to drive expression of *SU(Z)12* mRNA. To test this hypothesis, we cloned genomic fragments spanning both the upstream and downstream start sites in front of the luciferase cDNA. The promoter-reporter plasmids were then transfected into cells, and luciferase activity was monitored (Fig. 2*C*). We found that the upstream fragment, which corresponds to position −617 to +26 relative to the upstream start site mapped by primer extension, does mediate promoter activity and that this fragment functions bidirectionally. Interestingly, the first mammalian promoter shown to be regulated by E2F was the bidirectional *dhfr/msh3* locus (37). Other bidirectional E2F-regulated promoters include *thymidylate synthase* and *RanBP1/Htf9* (38, 39). We also note that in a recent screen for novel E2F-regulated promoters that identified 68 different loci, 15% of the promoters are clearly bidirectional (6). However, an analysis of the human genome suggests that a large percentage of human promoters, not just E2F target promoters, are bidirectional in nature (40). We have not further characterized the possibility that other transcripts may exist in the cell that are initiated in the opposite direction to the *SU(Z)12* mRNA. We found that the downstream fragment, which corresponds to position −243 to +76 relative to the downstream start site mapped by primer extension, also mediates promoter activity; in this case, only the forward direction is active. The relative promoter strength of the upstream *versus* downstream promoter fits well with the relative amounts of mRNA as determined by primer extension. Other E2F-regulated genes, such as human *E2F3*, *c-Myc*, human mitochondrial *glycerol phosphate dehydrogenase*, and human *uracil-DNA glycosylase* (41–44), have been shown to be controlled by dual promoters. Thus, we conclude that the *SU(Z)12* mRNA

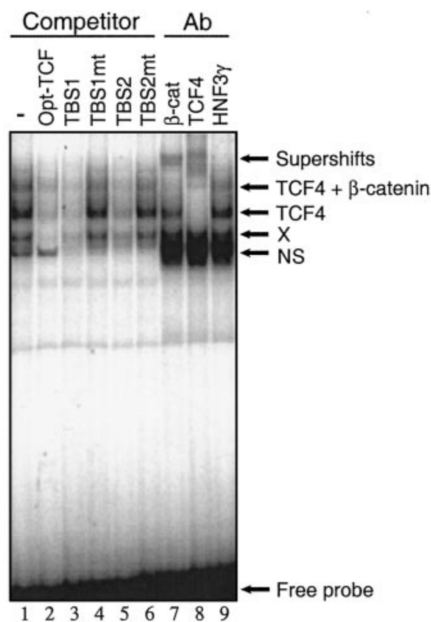

Fig. 3. Identification of TCF/β-catenin binding sites in the *SU(Z)12* promoter. An oligonucleotide containing a previously characterized consensus TCF site was used as a probe for the gel shift assays (36). The probe was incubated with HT29 whole cell extracts, alone or with a 10-fold excess of unlabeled probe (*Lane 1*), an oligonucleotide containing the proximal TCF binding site (*TBS1*) of the *SU(Z)12* promoter (*Lane 2*), an oligonucleotide containing a mutated TBS1 (*Lane 3*), an oligonucleotide containing the distal TCF binding site (*TBS2*) of the *SU(Z)12* promoter (*Lane 4*), or an oligonucleotide containing a mutated TBS2 (*Lane 5*). Antibodies that recognize β-catenin (*Lane 7*) or TCF4 (*Lane 8*) were incubated with the probe and whole cell extracts to identify the bands containing the TCF/β-catenin complexes. A control antibody, HNF3γ was added to the reaction in *Lane 9*. *NS*, a nonspecific band; *X*, a band representing an uncharacterized, but specific, DNA/protein complex.

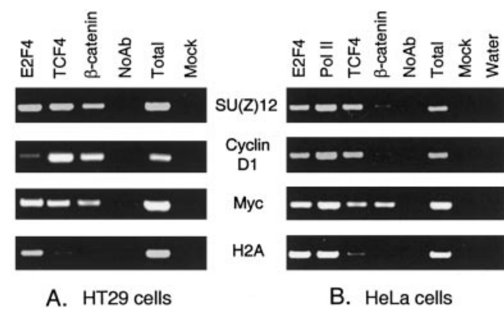

Fig. 4. ChIP analysis of the *SU(Z)12* promoter region. ChIP experiments were performed using the HT29 colon cancer cell line (*A*) or HeLa cells (*B*) and the indicated antibodies or a no-antibody control. The precipitated chromatin was monitored using primers specific for the *SU(Z)12*, *cyclin D1*, *c-Myc*, or *histone H2A* promoters. A mock immunoprecipitation reaction was also performed in which chromatin was omitted at the beginning of the experiment. Also, a control PCR reaction was performed in which only water (no immunoprecipitated sample) was added.

is driven from two promoters and that the bidirectional upstream promoter is the major promoter for *SU(Z)12* mRNA.

TCF and β-Catenin Are Recruited to the *SU(Z)12* Promoter. Sequence analysis of the *SU(Z)12* upstream promoter revealed two consensus binding sites for the TCF family of transciption factors, located at −206 and −776, relative to the upstream transcription start site. TCF family members do not contain transactivation domains. Rather, the coactivator β-catenin interacts with TCF family members and the TCF/β-catenin complex activates transcription. β-catenin is normally present in very low amounts in cells and is sequestered in membrane and/or cytoplasmic complexes (22). However, there are large amounts of nuclear β-catenin in colon cancer cells. We have examined the ability of the two consensus TCF sites in the *SU(Z)12* promoter to bind to TCF/β-catenin complexes using a gel mobility shift assay (Fig. 3). A previously characterized consensus TCF site was used as a probe. Incubation of this probe with extract prepared from HT29 colon cancer cells resulted in an upward shift of the probe, creating bands that are shown to be specific for binding of TCF factors attributable to competition by consensus (Fig. 3, *Lanes 2*, *3*, and *5*), but not mutated (*Lanes 4* and *6*) TCF sites. Inclusion of antibodies in the gel shift reaction identifies one band as free TCF4 and one band as a TCF4/β-catenin complex (*Lanes 7* and *8*); the other bands are likely to be other TCF family members alone or in complex with β-catenin. However, to date, we have not been able to identify which TCF family members compose the remaining bands. Importantly, inclusion of oligonucleotides corresponding to the distal and proximal TCF sites from the *SU(Z)12* promoter demonstrates that each of these sites has the potential to bind to TCF/β-catenin complexes *in vitro*.

Although gel mobility shift assays provide information as to whether a factor has the capability of binding to an isolated consensus site *in vitro*, they do not prove that a particular promoter is bound by a particular protein in living cells. However, the ChIP assay does provide a method to determine whether a particular promoter is bound by a specific transcription factor under biologically relevant *in vivo* conditions. To determine whether the *SU(Z)12* promoter is bound by TCF family members in living cells, we performed ChIP experiments using the HT29 colon tumor cell line (Fig. 4*A*). Antibodies to E2F4, TCF4, and β-catenin were used, along with a no-antibody control. As positive controls, we monitored occupancy of the *Myc* and *cyclin D1* promoters because both promoters have previously been shown to be E2F (17, 18) and TCF/β-catenin (15, 16) target genes. We also monitored the *histone H2A* promoter because it has been shown to be an E2F target gene (45) but has not been reported to be regulated by β-catenin. We used antibodies to E2F4 and TCF4, rather than antibodies to other E2F or TCF family members, because our preliminary experiments showed that these family members are easily detected on known target genes in colon tumor cell lines (data not shown). We found that, as expected, E2F4, TCF4, and β-catenin were bound to the *cyclin D1* and *c-Myc* promoters, whereas only E2F4 was bound to the *H2A* promoter (Fig. 4). As shown in Fig. 4*A*, we found that E2F4, TCF4, and β-catenin can be detected on the *SU(Z)12* promoter in HT29 cells. Interestingly, when similar experiments were performed using chromatin from HeLa cells (which are derived from a cervical cancer), we found that only the *c-Myc* promoter was bound by β-catenin (Fig. 4*B*). Unlike the *Myc* promoter, the *SU(Z)12* and *cyclin D1* promoters were only bound by β-catenin in the colon tumor sample. These results support

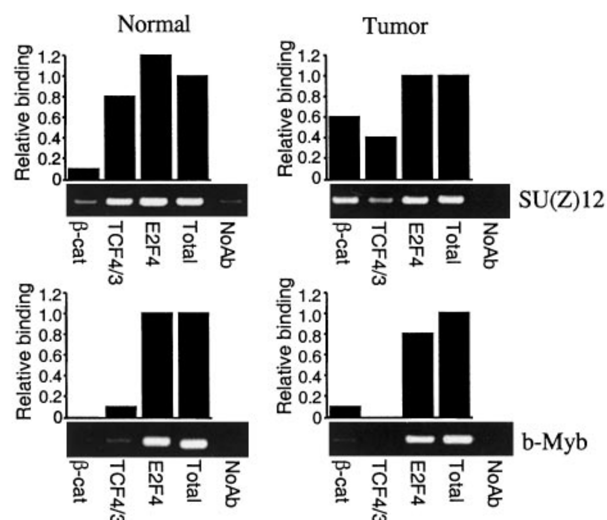

Fig. 5. Levels of β-catenin binding to the *SU(Z)12* promoter increase in colon tumors. ChIP experiments were performed using normal colon tissue or colon tumor tissue from the same patient, along with antibodies that recognize β-catenin, both TCF3 and TCF4, and E2F4, as well as a no-antibody control. Primers specific for the *SU(Z)12* and *b-Myb* (a well-characterized E2F target gene) promoters were used. The relative amount of binding of the different transcription factors was calculated by subtracting the slight signal in the no-antibody lane, then dividing the signal specific for the different factors by the signal obtained using a fixed aliquot of the input chromatin (*Total*).

the conclusion that the *SU(Z)12* promoter is bound by E2F and TCF/β-catenin complexes, and they also show that recruitment of β-catenin to a TCF target promoter can be influenced by cell type.

Levels of β-Catenin Binding to the *SU(Z)12* Promoter Increase in Colon Tumors. The ChIP assays performed above used cultured cells. If *SU(Z)12* is a biologically relevant β-catenin target gene, then we would expect that there should be little β-catenin bound to the promoter in normal colon tissue, but the levels would be increased in colon tumor samples. To test this hypothesis, we adapted the ChIP assay for use with surgical samples (details concerning the differences between assays performed using tissue culture cells *versus* surgical samples can be found at our web site).[7] We performed ChIP experiments using samples of matched normal colon and colon tumor from a cancer patient (Fig. 5). As a control, we monitored levels of E2F4. Although, as described above, the activity of E2F family members increases in certain tumors, the levels of E2F4 have not been shown to be influenced by neoplastic transformation. We found that the amounts of E2F4 bound to the *SU(Z)12* promoter and to the *b-Myb* promoter were similar in normal *versus* tumor tissues. In contrast, we found that levels of β-catenin bound to the *SU(Z)12* promoter increased in the tumor, as compared with the adjacent normal tissue. We have used several different matched normal *versus* tumor samples from various colon cancer patients. In all of the tumors in which we observed binding of β-catenin to the *Myc* promoter, we also found β-catenin bound to the *SU(Z)12* promoter (data not shown). Thus, we concluded that, in colon tumors, the *SU(Z)12* promoter is as effective in recruiting β-catenin as is the *Myc* promoter, a previously documented TCF/β-catenin target. Taken together, the gel shift analysis and the ChIP experiments indicated that the *SU(Z)12* promoter is occupied by TCF in normal cells and by TCF/β-catenin complexes in colon tumor cells.

We have noticed that the signal obtained using the TCF family member antibody is always slightly weaker in the tumor samples. This could be caused by a decreased accessibility of the antibody to the TCF family member caused, in turn, by the presence of the large β-catenin protein. Alternatively, in the tumor samples, TCF4 could be replaced by a different TCF family member. We have attempted to address this possibility by the use of antibodies to Lef-1, a TCF family member that has been reported to be increased in colon tumors (46). We did not enrich for the *SU(Z)12* promoter using the Lef-1 antibody in ChIP experiments (data not shown). However, we cannot conclude that Lef-1 did not replace TCF4 on the *SU(Z)12* promoter because none of the other TCF target promoters that we analyzed bound Lef-1, and thus we lacked a positive control.

Fig. 6. *SU(Z)12* mRNA is up-regulated in tumors. RT-PCR analysis was performed on RNA isolated from normal (*N*) colon and colon tumors (*T*) from five different patients, normal (*N*) breast and breast tumors (*T*) from four different patients, and normal (*N*) liver from mice not treated with DEN and three different mouse liver tumors (*T*) from mice treated with DEN, using primers specific for the human or mouse *SU(Z)12* mRNA. All of the samples were also analyzed by RT-PCR for *GAPDH* mRNA to ensure that the RNA was correctly quantitated and of high quality (30, 31).

SU(Z)12 mRNA Is Up-Regulated in Human Tumors. The experiments described above indicate that the *SU(Z)12* promoter is bound by high levels of β-catenin in colon tumors. If the recruitment of β-catenin to the *SU(Z)12* promoter is functionally significant, we would expect that levels of *SU(Z)12* mRNA would be increased in tumor types that are known to be associated with increased β-catenin activity. Therefore, we have investigated the expression of *SU(Z)12* in multiple different colon, breast, and liver tumors, all of which have been correlated with alterations in β-catenin activity (20, 47, 48). We found that *SU(Z)12* showed increased expression in five different human colon tumors, as compared with the normal colon tissue taken from the same patient (Fig. 6). These results, in combination with the analysis of four additional colon tumors (data not shown), indicated that *SU(Z)12* mRNA was more abundant in the tumor tissue in eight of nine colon cancer patients tested. In one of the patients, we found that *SU(Z)12* mRNA was high in both the normal and the tumor sample; it is possible that in this one

[7] Internet address: http://mcardle.oncology.wisc.edu/farnham/.

case, the normal sample was contaminated with tumor cells. Similarly, *SU(Z)12* mRNA was increased in four different human breast tumors. Because of the difficulty in obtaining human liver tumors samples, we have examined the expression of murine *SU(Z)12* in mouse liver tumors that were created by treatment of mice with the carcinogen DEN (see Ref. 31 for details). We found that *SU(Z)12* mRNA was increased in three different mouse liver tumors. Thus, increased expression of *SU(Z)12* mRNA occurs frequently in tumors derived from colon, liver, and breast.

Discussion

We have shown that the *SU(Z)12* promoter binds to TCF/β-catenin complexes *in vitro* and *in vivo* and that the levels of β-catenin binding to the *SU(Z)12* promoter increase in colon tumors, as compared with matched normal tissue. We also showed that the levels of *SU(Z)12* mRNA are increased in tumors known to be associated with high levels of nuclear β-catenin, namely tumors derived from colon, breast, and liver tissues. Thus, we conclude that *SU(Z)12* is a new β-catenin target gene whose expression is increased by recruitment of β-catenin in colon tumors. Interestingly, a recent report has shown that a portion of the *SU(Z)12* gene (called *JJAZ1* in that study) is fused to a gene from chromosome 7 in endometrial stromal tumors (2). In these tumors, most of the *SU(Z)12* coding sequences are fused in frame to the 5′ end of another protein called JAZF1. Therefore, the production of *SU(Z)12* mRNA in these endometrial stromal tumors is under the control of the *JAZF1* promoter, not the *SU(Z)12* promoter. Thus, *SU(Z)12* is activated by translocation in one tumor type (endometrial) and by β-catenin-mediated regulation in another tumor type (colon). Additional investigations are ongoing to understand the mechanisms by which the breast- and liver-tumor-specific increases in *SU(Z)12* mRNA are attained; it is possible that increased binding of β-catenin to the *SU(Z)12* promoter in these tumors types will also be observed.

Other previously identified β-catenin target genes include *fra-1*, *c-jun*, *Myc*, *matrilysin*, *cyclin D1*, *PGHS-2*, and *PPAR*δ (15, 16, 23–25). Most of these genes have been identified as β-catenin targets using cell culture experiments in which levels of β-catenin and/or APC have been artificially altered. Although we have shown that the *Myc*, *cyclin D1*, *jun*, and *PPAR*δ promoters are bound by β-catenin in colon tumors (Fig. 5 and unpublished data[8]), the other candidate target genes have not yet been validated using an *in vivo* binding assay. It is likely that aspects of the culture conditions used or even the process of establishing cell lines from the tumor samples may alter transcription factor binding profiles. Accordingly, we find that not all β-catenin target promoters show similar binding patterns in all cell types. For example, we detect β-catenin on the *Myc* promoter in both HeLa cells and in colon cancer cell lines. However, β-catenin is bound to the *cyclin D1* promoter only in the colon cancer cell line. Similar to the *cyclin D1* results, we found that β-catenin can be detected on the *SU(Z)12* promoter only in the colon tumor cells. We do not yet understand the molecular mechanisms responsible for mediating the specificity of recruitment of β-catenin to different promoters in different tissues. Possible models include the interchange of β-catenin with γ-catenin or the exchange of β-catenin with a transcriptional repressor such as groucho (49). It is also possible that tissue-specific recruitment of β-catenin may be enhanced by interaction with other site-specific transcription factors.

SU(Z)12 has homology to a group of transcriptional repressors called PcG proteins (3). PcG proteins, along with their counterparts called trithorax group proteins, which act as positive regulators of transcription, are involved in maintaining cellular identity during development and differentiation. The mechanism by which PcG and trithorax group proteins exert their control is via chromatin remodeling. Recent studies have shown that the *Drosophila* ESC-E(Z) chromatin remodeling complex consists of ESC, enhancer of Zeste [E(Z)], NURF-55, and SU(Z)12 (50). The human counterpart of the *Drosophila* ESC-E(Z) chromatin remodeling complex has also recently been purified. This complex, called EED-EZH2 (or *PRC*2), also contains SU(Z)12 (51, 54). Both the *Drosophila* and the human PcG complexes can methylate histone H3 on lysine 27. This methylation was shown to cause transcriptional repression of a target gene, confirming that SU(Z)12 is a component of a transcriptional repression/chromatin remodeling complex.

One hallmark of cancer is the loss of differentiation that occurs as normal cells undergo neoplastic transformation. PcG and trithorax group proteins have previously been found to be dysregulated in tumor cells of hematopoietic origin and it has been postulated that this dysregulation is important in causing and maintaining the neoplastic phenotype. A recent study has shown that EZH2, another component of the EED-EZH2 complex, is up-regulated in prostate cancers (52). Confirming the role of the EED-EZH2 complex in transcriptional regulation, ectopic expression of EZH2 led to transcriptional repression of a set of genes. Importantly, enforced down-regulation of EZH2 led to growth inhibition of a prostate cancer cell line. We have now shown that the mRNA of the PcG protein SU(Z)12 is increased in colon, breast, and liver cancers. We predict that SU(Z)12 target genes will also be deregulated in such cancers. Gene expression profiling of normal *versus* tumor colon tissue revealed 19 mRNAs that are increased at least 4-fold and 47 mRNAs that are decreased at least 4-fold in adenocarcinomas, as compared with normal tissue (53). It is interesting that a large number of genes were shown to be down-regulated in the tumors, given the fact that SU(Z)12 is part of a transcriptional repressor complex. The genes shown to be down-regulated in colon cancer are good initial candidates for SU(Z)12 target genes, as are the genes identified to be repressed by EZH2 (52).

Our future studies will focus on identifying SU(Z)12 target genes, using both a candidate gene approach and global screening methods. The studies that have demonstrated a requirement for EZH2 in the proliferation of prostate cancer cells suggest that targeting SU(Z)12 for inactivation in colon, breast, or liver tumor cells may also lead to the inhibition of proliferation. Therefore, we are also performing a functional

[8] Unpublished observations.

analysis of the SU(Z)12 protein with the goal of eventually designing a SU(Z)12-specific antitumor agent.

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