MOLECULAR AND CELLULAR BIOLOGY, Aug. 2008, p. 4963–4974 Vol. 28, No. 16
0270-7306/08/$08.00+0 doi:10.1128/MCB.00266-08

Feedback Regulation between Zipcode Binding Protein 1 and β-Catenin mRNAs in Breast Cancer Cells▽†

Wei Gu,* Amber L. Wells, Feng Pan,‡ and Robert H. Singer*

Department of Anatomy and Structural Biology and Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461

Received 15 February 2008/Returned for modification 14 April 2008/Accepted 7 May 2008

ZBP1 (zipcode binding protein 1) is an RNA-binding protein involved in many posttranscriptional processes, such as RNA localization, RNA stability, and translational control. ZBP1 is abundantly expressed in embryonic development, but its expression is silenced in most adult tissues. Reactivation of the ZBP1 gene has been reported in various human tumors. In this study, we identified a detailed molecular mechanism of ZBP1 transactivation in breast cancer cells. We show that β-catenin, a protein that functions in both cell adhesion and transcription, specifically binds to the ZBP1 promoter via a conserved β-catenin/TCF4 response element and activates its gene expression. ZBP1 activation is also closely correlated with nuclear translocation of β-catenin in human breast tumors. We further demonstrate feedback regulation by finding that ZBP1 physically associates with β-catenin mRNA in vivo and increases its stability. These experiments suggest that in breast cancer cells, the expression of ZBP1 and the expression of β-catenin are coordinately regulated. β-Catenin mediates the transcription of the ZBP1 gene, while ZBP1 promotes the stability of β-catenin mRNA.

ZBP1 (zipcode binding protein 1) belongs to a conserved family of RNA-binding proteins that contain four hnRNP K (KH) domains and two RNA recognition motifs (47). ZBP1 and its orthologues have been implicated in many aspects of RNA regulation, including intracellular RNA localization, stability, and translational control (5, 16, 21, 29, 34). A distinct role of ZBP1 is to establish cellular polarity in motile cells and developing neurons by facilitating asymmetric localization of β-actin mRNA and controlling its local protein synthesis (16). It has been suggested that ZBP1 identifies nascent β-actin RNA transcripts in the nucleus, and this determines the cytoplasmic fate of the mRNA (32, 33). Human ZBP1 (IMP1) also functions in the translational repression of insulin-like growth factor II mRNA (28, 32). In addition to regulation of mRNA localization and translation, mouse ZBP1 (CRD-BP) regulates the stability of c-*myc*, CD44, and β-TrCP1 mRNAs (25, 44). The effect of ZBP1 upon c-*myc* stability in vivo was elucidated in cord-blood-derived $CD34^+$ stem cells and ovarian carcinoma-derived ES-2 cells, where downregulation of the protein resulted in decreased c-*myc* mRNA and protein levels (19, 22). Moreover, stabilization of β-TrCP1 mRNA by ZBP1 may play a role in colorectal carcinogenesis by suppressing apoptosis via NF-κB activation (31). Therefore, by regulating mRNA turnover and translation of signaling and transcription factors, ZBP1 expression can affect cell survival and proliferation, contributing to embryonic development and tumor formation.

A large body of evidence has revealed ZBP1 as an oncofetal protein. ZBP1-deficient mice exhibited dwarfism, impaired gut development, and high perinatal lethality (12). ZBP1 expression is developmentally controlled in embryos of a variety of different organisms but disappears shortly after birth (37). While silenced in normal adult tissues, reexpression of ZBP1 has been observed in a high percentage of human tumors, including breast, ovarian, and colorectal tumors (48). The correlation of ZBP1 reexpression with tumorigenesis was revealed in transgenic mice in which targeted ZBP1 expression to mammary tissues induced mammary tumors, and the tumor incidence was positively correlated with increased ZBP1 expression levels (41). However, alternative functions of ZBP1 to repress proliferation and metastasis of cancer cells have also been reported (26, 45). These studies suggest the importance of ZBP1 regulation in cancer progression.

To date, little information is available regarding the underlying mechanism that leads to transcriptional regulation of the ZBP1 gene in cancer cells. Recent work has reported that CRD-BP, a member of the ZBP1 family, could be induced by expression of mutant β-catenin and TCF4 factors in 293T cells (31). However, the molecular mechanism of how the gene is activated in response to β-catenin/TCF4 expression has not been determined. To reveal the molecular basis for the characteristic expression of the gene in human cancers, we have cloned the ZBP1/CRD-BP promoter and functionally characterized its activity in mammalian breast cancer cell lines. We demonstrate that β-catenin, a protein involved in transactivation of many oncogenes (13, 40), specifically interacts with a conserved element within the ZBP1 promoter, through which it activates transcription. We also show that ZBP1, in turn, is able to bind to β-catenin mRNA and regulate its expression posttranscriptionally. Our study describes a novel feedback

* Corresponding author. Mailing address: Department of Anatomy and Structural Biology and Department of Cell Biology, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, NY 10461. Phone for W. Gu: (718) 430-8715. Fax: (718) 430-8697. E-mail: wgu@aecom.yu.edu. Phone for R. H. Singer: (718) 430-8646. Fax: (718) 430-8697. E-mail: rhsinger@aecom.yu.edu.

‡ Present address: Molecular Neurobiology Program, Skirball Institute, New York University Medical Center, New York, NY 10016.

† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 19 May 2008.

loop whereby β-catenin and ZBP1 can regulate each other's expression in mammalian breast cancer cells.

MATERIALS AND METHODS

Cell lines, cell culture, transfection, and luciferase assays. MTC cells were cultured in minimal essential medium α with 5% fetal bovine serum as previously described (46). T47D and 293T cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. MCF 10A, a human nontumorigenic breast epithelial cell line was purchased from ATCC (CRL 10318) and cultured as instructed. For transfection assays, MTC and T47D cells were initially cultured and grown overnight to 60 to 80% confluence. Cells were harvested and transiently cotransfected with a mixture containing 1.0 μg of the individual luciferase reporter plasmids and 0.1 μg of an internal control of *Renilla* luciferase plasmid using a Nucleofector (Amaxa) following the manufacturer's instructions. After transfection, the cells were placed into 96-well plastic plates and cultured for 16 to 24 h. Cellular luciferase activities were measured using the Promega luciferase assay system. Where indicated, cells transfected with a simian virus 40 (SV40) pG2 vector or a promoterless pG2 vector were used as a positive or a negative control.

Western blotting. Antibodies used in Western blotting were monoclonal antibodies against β-actin (Sigma-Aldrich), β-catenin (Chemicon International), acetylated histone 2B, and polyclonal ZBP1 antibody (lab generated). The Western blots were developed using the ECL enhanced chemiluminescence method (Amersham Biosciences) or using an Odyssey detection instrument (LI-COR Inc). Band intensities were determined by densitometry or NIH ImageJ software.

Northern blot analysis. Total RNAs were isolated from cultured MTC, T47D, and 293T cells, rat embryos, and adult tissues using an RAeasy minikit (Qiagen Inc.). Ten micrograms of total RNAs were electrophoresed in 1% agarose gels and transferred to a polyvinylidene difluoride membrane. A 400-bp cDNA encoding the N terminus of rat ZBP1 was ^{32}P labeled using the Stratagene random labeling kit and used for hybridization.

RNase H assays. The assays were performed as described previously (11). Briefly, RNA aliquots (15 μg) were denatured in 80% formamide at 65°C for 10 min and annealed with 0.5 μg of specific oligonucleotide(s) complementary to the ZBP1 mRNA for 90 min at 50°C in a buffer containing 10 mM PIPES (pH 6.4), 40% formamide, 0.4 M NaCl, and 1 mM EDTA. The hybridized nucleic acids were ethanol precipitated, dried, resuspended in 100 μl of RNase H digestion buffer (20 mM Tris [pH 7.4], 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM dithiothreitol), and incubated with 6 U of RNase H for 45 min at 37°C. The samples were extracted twice with phenol-chloroform, and the cleaved RNAs were precipitated with ethanol. The precipitates were electrophoresed in 1% agarose gels and analyzed by Northern blotting.

Constructs. Various lengths of the ZBP1 promoter were PCR amplified from rat genomic DNA and cloned upstream of the firefly luciferase gene of pGL2 plasmid at the SacI and HindIII sites. Sequencing analyses were performed on the ZBP1 constructs to ensure no mutations were involved during the PCR and cloning processes.

RT and PCR detection of mRNA. First-strand cDNA was synthesized in a volume of 20 μl containing 1 μg of total RNA isolated from cultured cells, 2 pM of gene specific primers, and 200 U of SuperScript III reverse transcriptase (Invitrogen). Nested-PCR amplification was performed in a 50-μl volume containing 2 μl of the reverse transcription (RT) reaction mixture, 0.3 U of Accuprime *Taq* polymerase (Invitrogen), and 10 pM each of a selected primer pair for the genes of interest. The PCR was run for 20 to 30 cycles with 0.5 min of denaturing at 94°C, 0.5 min of annealing at 55°C, and 0.5 min of DNA synthesis at 68°C and then a 5-min extension at 68°C. Control detection was performed with the reaction without the RT step. The PCR products were resolved in 1% agarose gels, and the band intensities were normalized to their respective β-actin band intensities using NIH ImageJ software. Experiments were repeated at least two times independently, and the reproducibility was verified.

Preparation of nuclear extracts. MTC and T47D cells were scraped from the cultured dishes and washed twice in ice-cold phosphate-buffered saline (PBS). Nuclear extracts used for DNA mobility shift and in vitro transcription assays were prepared as described previously (4). Protein concentrations of nuclear extracts were determined by the Pierce protein assay kit at a range from 3 to 5 mg/ml.

DNA gel mobility shift assays. Sense and antisense DNA oligonucleotides (−578 to −602 of the ZBP1 promoter) containing the putative β-catenin/TCF4 binding site (CTTTG-TC) were synthesized and annealed to form a double-stranded DNA fragment. The DNA fragment was ^{32}P-end labeled with T4 DNA kinase (Promega) and gel purified. DNA gel shift assays were performed using labeled DNA fragment (10,000 cpm) and nuclear extracts as previously described (49).

RNA gel mobility shift assays. ^{32}P-labeled RNA fragments (positions +118 to +232 and +1583 to +1712 and a 240-nucleotide [nt] 3′ untranslated region [UTR] of β-catenin mRNA) were in vitro transcribed using a T7 RNA polymerase. RNA gel shift assays were performed using recombinant ZBP1 and labeled RNA fragments as previously described (33).

In vitro transcription. In vitro transcription was performed as described previously (38). Briefly, aliquots of nuclear extracts (5 to 8 μl) of cultured MTC or T47D cells were incubated with 1.0 μg of DNA templates in 20 μl of reaction mixture containing 10 mM HEPES (pH 7.9), 26 mM KCl, 6 mM $MgCl_2$, 0.6 mM each of ATP, CTP, and GTP and 36 μM UTP (Invitrogen), 5 μCi of [^{32}P]UTP (Amersham Corp.), 0.5 μl of RNase inhibitor (Roche), and 3% glycerol. The reaction mixtures were incubated at 30°C for 45 min and were terminated by the addition of 0.3 ml of stop buffer (20 mM Tris [pH 7.5], 0.25 M NaCl, 1% sodium dodecyl sulfate, 5 mM EDTA, 50 μg of proteinase K, and 20 μg of *Escherichia coli* tRNA). After incubation at 37°C for 30 min, the reaction mixtures were extracted with phenol-chloroform and precipitated with ethanol. The precipitates were washed in 70% ethanol, air dried, and dissolved in 6 μl of 98% formamide loading buffer. After heating at 90°C for 5 min, the RNA samples were electrophoresed in 6% denaturing polyacrylamide gels and visualized by autoradiography. The transcription activities of the ZBP1 promoter constructs were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Immunofluorescence, microscopy, and imaging. Cells cultured on glass coverslips were permeabilized with 0.5% Triton X-100 and fixed with 3% formaldehyde in PBS. The coverslips were incubated with primary antibodies against the proteins of interest followed by incubation with Cy3 or Cy5 fluorescently labeled secondary antibodies. Cells were viewed under an Olympus BX61 epifluorescence microscope equipped with an Olympus PlanApo ×60, 1.4-NA oil objective. Images were acquired with a Roper coolSNAP HQ cooled charge-coupled device camera (Roper Scientific) operated by the IPlab software package (Scanalytics, Inc).

siRNA experiments. Small interfering RNAs (siRNAs) targeting rat β-catenin and TCF4 mRNAs were designed by software packages of Dharmacon Thermo Fisher Scientific and synthesized by Invitrogen, Inc. The duplexed siRNA sequences against β-catenin were sense sequence 5′-GAGGGCUUGUUGGCCA UCUUUAAAU and antisense sequence 5′-AUUUAAAGAUGGCCAACAAG CCCUC. siRNA sequences against TCF4 mRNA were sense sequence 5′-UGA AGACCGUUUAGAAAGATT and antisense sequence 5′-UCUUUCUAAAC GGUCUUCATT. The siRNA used for knocking down the rat ZBP1 gene was reported previously (18). One day before transfection, cells were seeded such that they were 30 to 50% confluent the next day. Cells were transfected with 100 nM of siRNA using Lipofectamine 2000 reagent (Invitrogen) in Opti-MEM medium (Invitrogen) at 37°C in a 5% CO_2 atmosphere overnight. After removing the siRNA medium, fresh culture medium without siRNA was added and cells were cultured for an additional 24 to 48 h. Control cells were treated with Lipofectamine 2000 reagent alone or with a mock, scrambled siRNA. Cells were harvested after treatment and used for analyzing β-catenin, TCF4, ZBP1, and β-actin expression by RT-PCR or Western blotting. Where indicated, MTLn3 and MTLn3-ZBP1 cells were treated with 5 μM actinomycin D and were analyzed for β-catenin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and α-tubulin expression by RT-PCR.

ChIP. MTC cells were scraped from the cultured dishes and washed twice in ice-cold PBS. Chromatin immunoprecipitation (ChIP) was performed as described previously (4). For PCR after ChIP, 1 to 2 μl of a 50-μl DNA extraction from ChIP and 25 to 30 cycles of amplification were used.

Human breast tumor samples and immunohistochemical analysis. Human breast tumor samples were purchased from US Biomax, Inc. Each sample contained two paraffin-embedded tumor sections. Immunohistochemical analysis was performed as described previously (1). The samples were stained with primary antibodies against β-catenin and ZBP1 followed by Cy3- and Cy5-conjugated secondary antibodies.

Isolation of ZBP1 mRNP complexes, RNA extraction, and microarray analysis. MTLn3 and MTLn3-ZBP1 (MTLn3 stable cell line constitutively expressing FLAG-tagged ZBP1) cells were lysed in ice-cold lysis buffer (10 mM HEPES [pH 7.8], 40 mM NaCl, 10 mM KCl, 0.5% NP-40, 0.5 μg/ml phenylmethylsulfonyl fluoride, 1× protease inhibitor mixture [Roche]). Nuclei were pelleted (3,500 rpm for 4 min at 4°C), and supernatants were subjected to an additional step of high-speed centrifugation (14,000 rpm for 60 min at 4°C). The supernatants, after adding 250 U of RNase inhibitor (Roche Applied Science)/ml of lysis solution, were incubated with FLAG-specific monoclonal antibody M2 covalently coupled to agarose beads (Sigma), which were preincubated with bovine serum albumin and yeast tRNA and washed three times in lysis buffer. The suspension was rotated for overnight at 4°C, followed by a short centrifugation to remove the supernatants. The beads were extensively washed in lysis buffer followed by

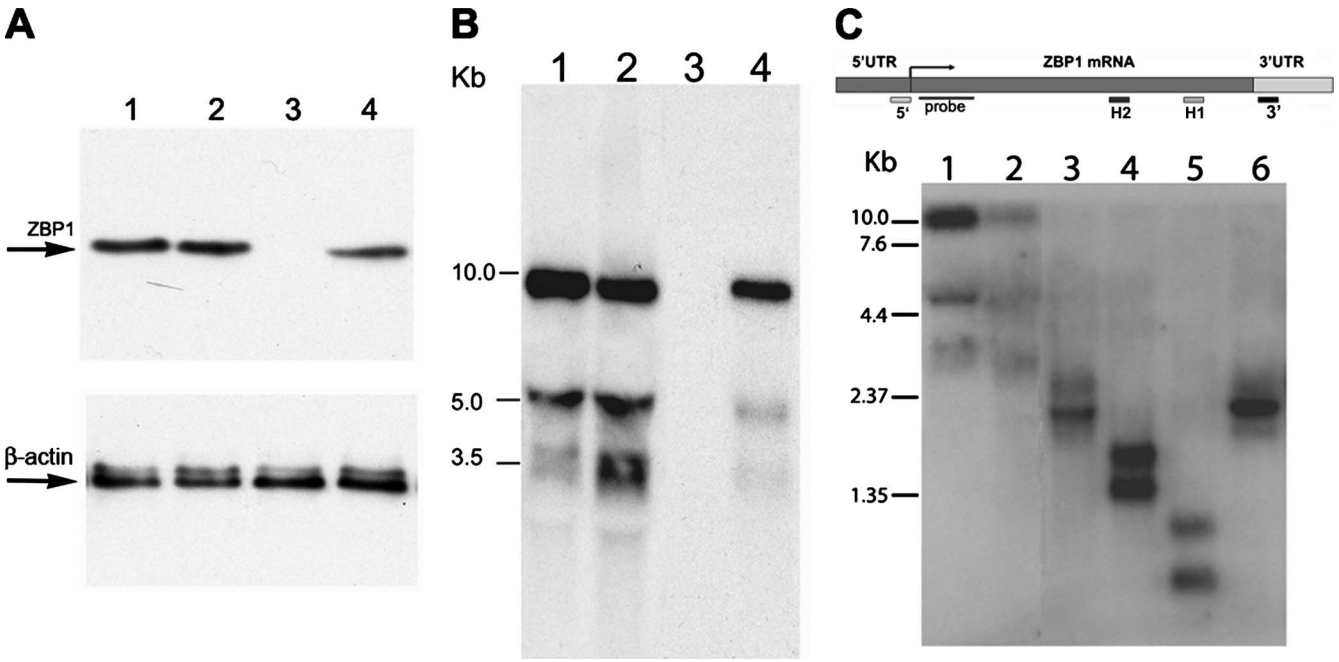

FIG. 1. ZBP1 expression in mammalian breast carcinoma cells. (A) Analyses of ZBP1 and β-actin expression in rat embryos and the MTC cell line, rat adult livers (lanes 1, 2, and 3, respectively), and the human T47D cell line (lane 4). The arrows indicate the proteins detected with antibodies against ZBP1 and β-actin proteins. (B) ZBP1 mRNA expression in rat embryos (lane 1), MTC cells (lane 2), rat adult liver (lane 3), and T47D cells (lane 4). The molecular sizes of the ZBP1 transcripts are marked to the left. (C) RNase H assays to determine the structural variations of ZBP1 mRNAs. A schematic diagram indicating the positions of the oligonucleotides and the cDNA probe used in RNase H experiments is shown in the upper panel. Aliquots of total RNAs from MTC cells were hybridized to nonspecific oligonucleotides (lane 1), 5′ oligonucleotides (lane 2), 3′, H1, or H2 oligonucleotides (lanes 3, 4, and 5, respectively), and combined 5′ and 3′ oligonucleotides (lane 6). Hybridized RNA samples were subjected to RNase H digestion followed by Northern hybridization with a cDNA probe encoding the N-terminal portion of the protein. The molecular sizes of RNAs are marked to the left.

adding 1 ml of TRIzol. RNAs associated with FLAG-ZBP1 were extracted in TRIzol as described by the manufacturer (Invitrogen). DNA microarray experiments with immunoprecipitated RNA were performed using a Rat Genome 230 Plus 2.0 array, and data were analyzed at the Center for Functional Genomics at University of Albany.

RESULTS

Characterizing ZBP1 transcripts. We examined ZBP1 protein and mRNA expression in rat embryonic and adult tissues, as well as in cultured rat MTC cells and human T47D cells. Western blots demonstrated that ZBP1 was expressed in rat embryonic tissues, MTC cells, and T47D breast cancer cells (Fig. 1A, lanes 1, 2, and 4, respectively), but not in adult livers. Probing the blot with antibodies against β-actin indicated that undetected ZBP1 was not the result of insufficient protein loading (Fig. 1A, lower panel). We then examined the levels of ZBP1 mRNAs by Northern assays which detected three sizes of ZBP1 transcripts with molecular sizes of approximately 10, 5, and 3.5 kb in MTC cells, rat embryos, and T47D cells (Fig. 1B, lanes 1, 2 and 4, respectively). Consistent with the Western blotting result, ZBP1 mRNAs were not expressed in rat adult livers (Fig. 1B, lane 3). Among the ZBP1 transcripts, the 10-kb mRNA appeared as the major transcript. Since the anti-ZBP1 antibody only detected a single protein (Fig. 1A), it is unlikely that the mRNAs encoded other homologous proteins. ZBP1 was also not detected in rat adult mammary tissues and MCF 10A, a nontumorigenic human breast cell line, by Western blotting and RT-PCR (see Fig. S1 in the supplemental material). These results suggest that ZBP1 expression is transcriptionally controlled. Multiple sizes of ZBP1 mRNAs were also detected in other species, including mice (30) and chickens (31a), reflecting the high evolutionary conservation of this gene family.

To define the structural differences among the ZBP1 mRNAs, we isolated the RNA from MTC cells and utilized a combined assay of RNase H digestion and Northern blotting (Fig. 1C). We hybridized ZBP1 mRNAs with specific oligonucleotides complementary to different positions within the coding sequence, digested the mRNAs into 5′ and 3′ regions with RNase H, and analyzed them by Northern hybridization. As shown in Fig. 1C, separating the 5′ UTR from ZBP1 mRNAs resulted in small size changes in comparison with that of a control RNA sample (lanes 1 and 2), indicating that the 5′ UTR contributed little to the sizes of the ZBP1 mRNAs. However, detaching the 3′ UTR from ZBP1 mRNAs yielded two mRNA bands in which the slower-migrating band appears to be 400 nt longer than the faster-migrating transcript (lane 3). Similar results were obtained when targeting H1 and H2 oligonucleotides to the 3′ coding regions of the ZBP1 mRNAs (lanes 4 and 5). However, hybridization of ZBP1 mRNAs with combined 5′ and 3′ oligonucleotides that removed both the 5′ and 3′ UTRs generated only one mRNA transcript with the size of approximately 1.8 kb (lane 6). These data suggest that the three ZBP1 mRNAs are transcribed from two alterna-

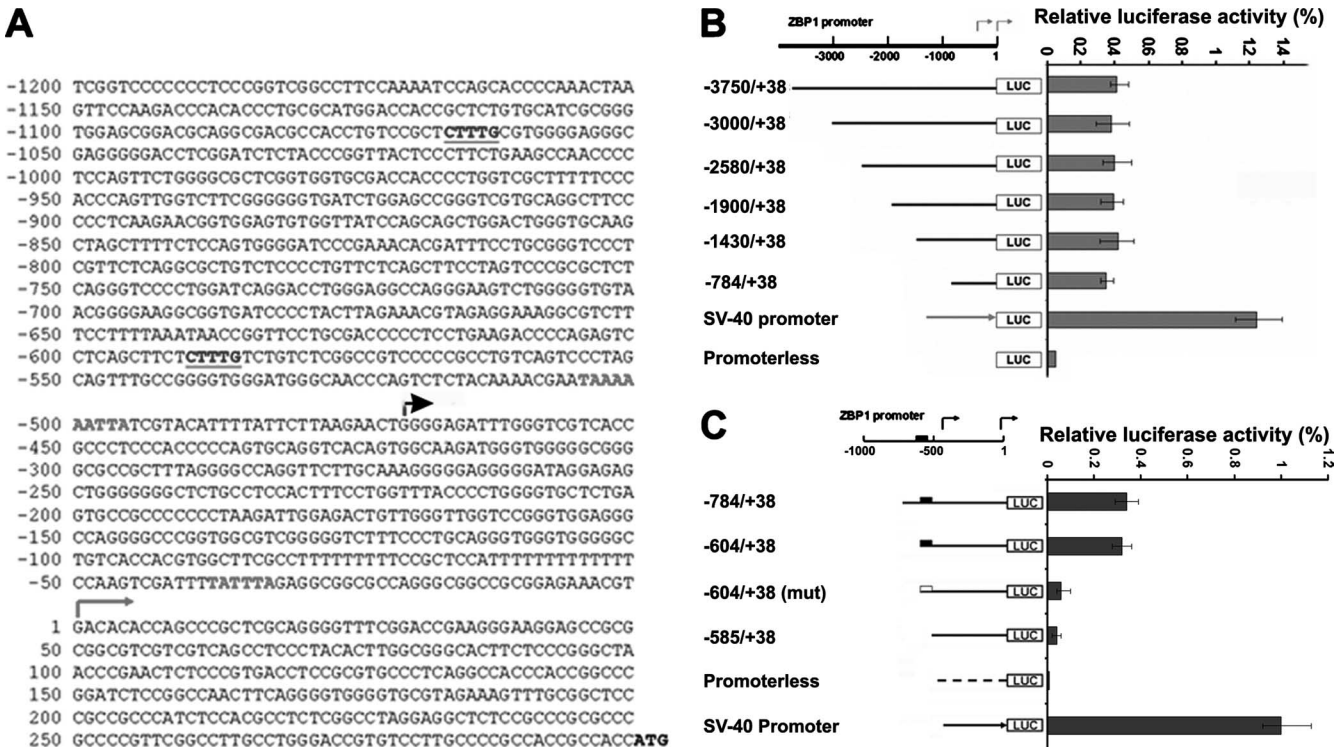

FIG. 2. Sequence and function of the ZBP1 promoter. (A) ZBP1 mRNAs are transcribed from two alternative initiation sites. The transcription initiation sites of the ZBP1 promoter were determined using 5′-RNA ligase-mediated RACE experiments. Bases are numbered with respect to the major proximal transcription initiation site (designated as +1), which is indicated with an arrow. The alternative distal transcription site is marked with an arrowhead (−471). The ATG translation start codon is denoted. The nucleotides comprising the two unconventional TATA boxes are indicated in boldface. The putative elements for β-catenin/TCF4 binding are underlined. (B and C) Analyses of ZBP1 promoter activities. (Left) Schematic representation of the luciferase reporter constructs (LUC) being driven by 5′-nested deletions of the ZBP1 promoter. Relative positions of the promoter region in each construct are marked. The arrows indicate the two putative transcription initiation sites. The dark blocks denote the position of the proximal site for putative β-catenin/TCF4 binding. The open white block indicates the mutation (mut) of the proximal site (CTTTG to CTTCG). Dashed lines indicate the deletion sequences of the ZBP1 promoter. Different lengths and deletions of the ZBP1 promoter were subcloned upstream of the firefly luciferase reporter gene in the pGL2 plasmid. (Right) Constructs with the ZBP1 promoter and an internal control *Renilla* luciferase plasmid were cotransfected into cultured MTC cells. The firefly luciferase activity from each transfection is normalized to the *Renilla* luciferase activity, and the relative luciferase activity is represented as a percentage of that of the pSV-40 construct. The results are the means of a total of three independent experiments carried out in triplicates.

tive initiation sites; the distance between the two sites is about 400 nt.

Multiple ZBP1 mRNAs are transcribed from two alternative sites. We used 5′ rapid amplification of cDNA ends (RACE) experiments to determine the transcription initiation sites of the ZBP1 gene. We isolated total RNA from cultured MTC cells and performed RT followed by nested-PCR amplification. The nested PCR generated two major cDNA fragments encoding the 5′ UTRs of ZBP1 mRNAs (data not shown). The size difference between the two PCR fragments was about 400 bp, which was consistent with the RNase H experiments. The PCR products were cloned and sequenced. Sequencing analyses of 12 independent clones of shorter PCR products revealed transcriptional initiation sites. The major site was initiated from the guanine residue located 300 bp upstream of the ATG start codon. Accordingly, this base was designated bp +1 (Fig. 2A). Sequencing the clones of longer PCR products revealed an alternative transcription site at 771 bp (bp −471) upstream of the ATG codon (Fig. 2A).

Analysis of the 1.2-kb promoter region indicated that transcription of the ZBP1 gene appeared to be initiated from two noncanonical TATA boxes. The promoter region was highly conserved, with 91% sequence identity between rat and mouse genes and 78% sequence identity between rat and human genes. Moreover, a number of consensus elements for transcription factors including SP1, CREB, and AP-2 were identified within this region (not shown). Most of these putative sites were located between the two transcription initiation sites. Noticeably, the region upstream of the distal initiation site contained two putative elements of the CTTTG motif, which is a target for β-catenin-mediated TCF/LEF binding (14). Overexpression of a mutant β-catenin and TCF4 showed effective transactivation of the ZBP1 gene in human 293T cells (31). We investigated whether breast cancer cells use the same β-catenin/TCF4 pathway to regulate ZBP1 expression.

ZBP1 promoter-driven reporter plasmids were effectively expressed in breast cancer cells. To determine the functional promoter region of the ZBP1 gene required for its transcriptional activity, we carried out in vivo luciferase reporter assays. We generated various lengths of the 5′ flanking region of the rat ZBP1 gene by PCR amplification and inserted them into the 5′ end of a firefly luciferase gene in a pGL2 reporter vector

(Promega). A promoterless parent pGL2 vector and a pGL2 vector under the control of an SV40 promoter/enhancer were used as negative and positive controls for luciferase activity, respectively (Fig. 2B, left). The reporter constructs were transiently cotransfected with an internal control, a *Renilla* luciferase plasmid, into cultured MTC cells. The firefly luciferase activity was determined for each transfection and normalized with respect to corresponding *Renilla* luciferase activity. The luciferase activity driven by the SV40 promoter was high, whereas the activity was barely detected in cells transfected with a promoterless vector (pGL2-Basic). The luciferase reporters driven by various lengths of the ZBP1 promoter were relatively active in the cells. Sequential deletion of the ZBP1 promoter from bp −3750 to bp −1430 did not significantly change reporter activity, which suggested that the 1.43-kb 5′ flanking region was sufficient for ZBP1 promoter activity (Fig. 2B, right). The 1.43-kb promoter region contains two putative sequence elements of CTTTG for β-catenin/TCF4 transcription factor binding at bp −590 (proximal binding site) and −1067 (distal binding site). Deleting the distal binding site (−784 to +38) resulted in a slight decrease in luciferase activity, indicating that the distal binding site was not essential for the promoter activity. T47D cells transfected with the constructs were also capable of producing luciferase activity with a similar efficiency compared to MTC cells (see Fig. S2 in the supplemental material). These results indicated that reporters under the control of the rat ZBP1 promoter could be effectively expressed in both rat and human breast cancer cells.

To evaluate whether the proximal CTTTG-TC *cis* element was required for ZBP1 promoter activity, we constructed luciferase reporters under the control of the ZBP1 promoter with or without the proximal element (Fig. 2C, left). Transfection of a ZBP1 promoter construct (−585 to +38) that deleted the CTTTG-TC proximal β-catenin/TCF binding elements significantly reduced the expression efficiency of the reporter by nearly 80%. Decreasing luciferase activity was also observed in the reporter where the CTTTG-TC element of the ZBP1 promoter was mutated (Fig. 2C, left, CTTTG mutated [underlined] to CTTCG). These data indicate that the proximal β-catenin/TCF4 binding site was essential for the proper activity of the ZBP1 promoter.

Transactivation of the ZBP1 gene correlates with nuclear accumulation of β-catenin. ZBP1 expression was induced in 293T cells in response to a β-catenin/TCF4-mediated pathway (31). To investigate whether β-catenin/TCF4 activates ZBP1 expression in mammalian breast carcinoma cells, we examined cellular levels and distribution of β-catenin/TCF4 in cultured MTC and T47D cells. RT-PCR analyses indicated substantial levels of β-catenin and TCF4 mRNAs in both cell lines, while in control 293T cells, levels of β-catenin mRNA were lower and almost no TCF4 mRNA was detected (Fig. 3A). Western blots revealed that β-catenin was abundant in nuclear extracts of MTC and T47D cells (Fig. 3B). Other members of the TCF/LEF family, such as TCF1, TCF3, and LEF1 (2, 42, 43) displayed low levels of expression (data not shown), indicating that TCF4 was the major protein with which β-catenin associated in the cells tested. An indirect immunofluorescence assay, which was performed to further determine the cellular distribution of β-catenin, showed that in addition to localizing at the cell-cell contacts, it was highly accumulated within the nuclei of

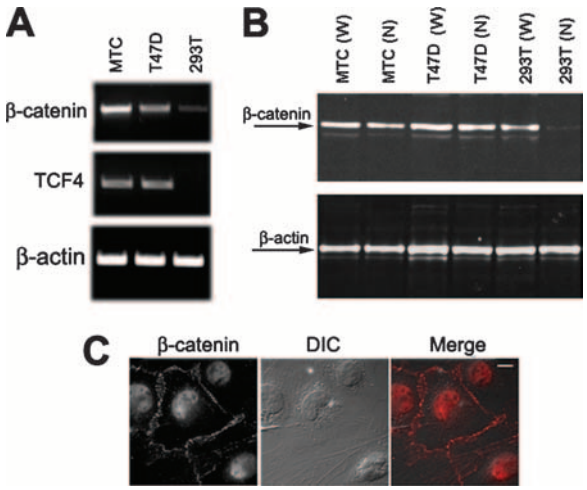

FIG. 3. Expression of β-catenin/TCF4 in breast cancer cells. (A) Equal amounts (1 μg) of total RNAs isolated from cultured MTC, T47D, and 293T cells were subjected to RT-PCR analyses with gene-specific primers to β-catenin, TCF4, and β-actin mRNAs. (B) Western blot showing the levels of β-catenin and β-actin in whole-cell extracts (W) and nuclear extracts (N) of MTC, T47D, and 293T cells. The arrows indicate the detected β-catenin and β-actin proteins. (C) Immunofluorescence showing the cellular localization of β-catenin in MTC. An average of 100 cells was examined. β-Catenin was found predominantly in cell nuclei (100%). Bar, 10 μm.

MTC cells (Fig. 3C). β-Catenin was also associated with the nucleus of T47D cells, but this localization appeared oriented to the nuclear periphery (see Fig. S3 in the supplemental material). These results suggest a potential association of nuclear β-catenin with the transactivation of the ZBP1 gene.

An association between ZBP1 expression and nuclear translocation of β-catenin was also observed in human breast tumors. This observation was from immunohistochemical analyses using antibodies against β-catenin and ZBP1 in five paraffin-embedded human breast tumor samples (Biomax, Inc.). Representative immunohistochemical profiles are shown in Fig. S4 in the supplemental material. In three of the five tumor samples, β-catenin (red) was found to accumulate within the nuclei of 83% of tumor cells, in which 60% demonstrated ZBP1 expression (green) (upper panel). In two tumor tissue samples, β-catenin did not appear in the cell nuclei and ZBP1 expression was undetectable (see Fig. 7, lower panel). These data suggest that in human breast tumors, β-catenin signaling could play a role in induction of ZBP1 expression.

Targeted knockdown of β-catenin or TCF4 downregulates ZBP1 expression. To test whether transcription of the ZBP1 gene could be mediated by the β-catenin/TCF4 pathway, we performed siRNA experiments (6, 7) in MTC cells to knock down β-catenin or TCF4 and used Western blotting and RT-PCR analyses to examine the consequent ZBP1 expression. siRNA targeted to β-catenin mRNA led to substantial downregulation of β-catenin to 26% of the wild-type level (Fig. 4A and B) and β-catenin mRNA to 31% of the wild-type level (Fig. 4C and D) after 48 h of transfection. Knockdown of β-catenin function resulted in a significant decrease of ZBP1 to 47% (Fig. 4A and B) and ZBP1 mRNA to 49% (Fig. 4C and D). Since TCF4 was the major member of the TCF/LEF family associated with β-catenin in MTC cells, we also targeted

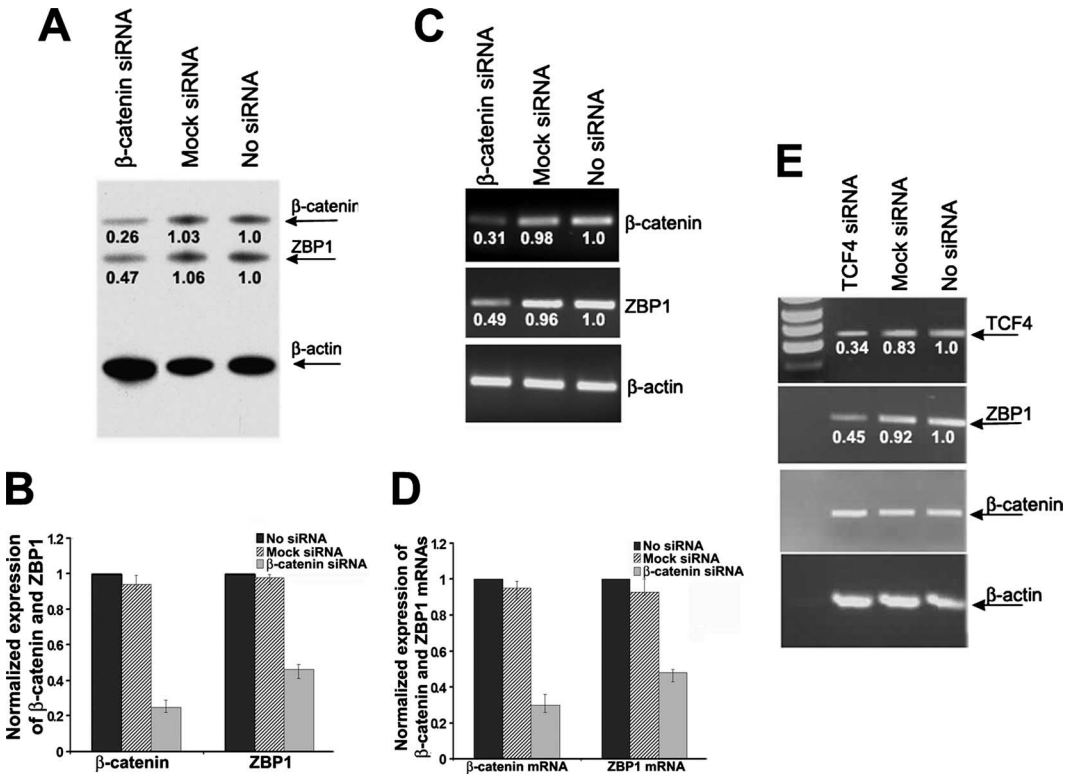

FIG. 4. Knockdown of β-catenin or TCF4 downregulates ZBP1 expression. (A) A representative Western blot shows the expression of β-catenin, ZBP1, and β-actin in MTC cells transfected with siRNA to β-catenin. Numbers below the bands reflect the decrease (*n*-fold) of protein expression after normalizing to β-actin. The arrows indicate the detected proteins. (B) Relative levels of β-catenin and ZBP1 were normalized and averaged to β-actin from three independent experiments. (C) RT-PCR analysis of β-catenin, ZBP1, and β-actin transcripts in MTC cells transfected as indicated. Numbers below the bands indicate the reduction (*n*-fold) of β-catenin and ZBP1 mRNAs. (D) Relative levels of β-catenin and ZBP1 mRNAs were normalized and averaged to β-actin mRNA from three independent experiments. (E) Effects of TCF4 downregulation on ZBP1 expression. MTC cells were transfected either with TCF4-specific or scrambled siRNA for 2 days and were subjected to RT-PCR. Numbers below the bands reflect relative levels of TCF4 and ZBP1 transcripts normalized to β-actin mRNA after transfection.

knockdown of TCF4 function and utilized RT-PCR to analyze the corresponding ZBP1 expression. TCF4-specific siRNA could efficiently downregulate TCF4 expression (34%), while a mock siRNA only slightly affected its expression (83%). As a consequence of TCF4 knockdown, ZBP1 expression in the cells decreased to 45% (Fig. 4C). Expression of control β-actin mRNA was not affected in the experiments performed (Fig. 4A, B, and C). These experiments suggest that both β-catenin and TCF4 are required for ZBP1 gene expression in MTC cells.

The proximal CTTTG-TC element of the ZBP1 promoter associates with β-catenin in vivo and is required for transcriptional activity of the ZBP1 promoter in vitro. β-Catenin facilitates the binding of TCF/LEF family members to the consensus sequence element CTTTG-A(T)-A(T) in the promoter region of its target genes, which regulates their gene expression (8, 14). β-Catenin associates with the promoter region of the ZBP1 gene containing a complementary CTTTG-TT element (−1333 to −1607 upstream of the ATG codon) in human 293T cells (31); however, this element was not found in the promoters of mouse and rat ZBP1 genes. The promoter of the rat ZBP1 gene contains two putative CTTTG *cis* elements located at positions −588 (CTTTG-TC; proximal site) and −1065 (CTTTG-CG; distal site) upstream of the transcription initiation site (Fig. 2A). The proximal element is conserved in the rat, mouse, and human ZBP1 promoters, but the distal element is not present in the human promoter. Both elements contain mismatches at their flanking nucleotides that could affect their binding affinity to β-catenin. To test whether β-catenin associated with the ZBP1 promoter via the putative elements, we performed ChIP and PCR analyses. Cultured MTC cells were subjected to ChIP with equal amounts of antibodies against acetylated histone or β-catenin. Two primer pairs were designed to amplify the ZBP1 promoter containing the putative distal or proximal β-catenin/TCF4 sites with expected sizes of 260 bp (−1196 to −940) or 250 bp (−619 to −370), respectively (Fig. 5A, distal site, lanes 1 to 3, or proximal site, lanes 4 to 6, respectively). An additional primer pair to amplify a 250-bp chromatin fragment 2 kb upstream of the transcription initiation site of the ZBP1 gene was used as a negative control (Fig. 5A, no binding site, lanes 7 to 9). As expected, the anti-acetylated histone antibody was able to precipitate all three chromatin fragments of the ZBP1 promoter (lanes 2, 5, and 8, respectively). The β-catenin antibody was also able to effectively pull down the ZBP1 promoter containing the proximal CTTTG-TC element (lane 6). In contrast, precipitation of the promoter region containing the distal CTTTG-CG element with the antibody was less efficient, presumably resulting from the 2-nt mismatch with the consensus

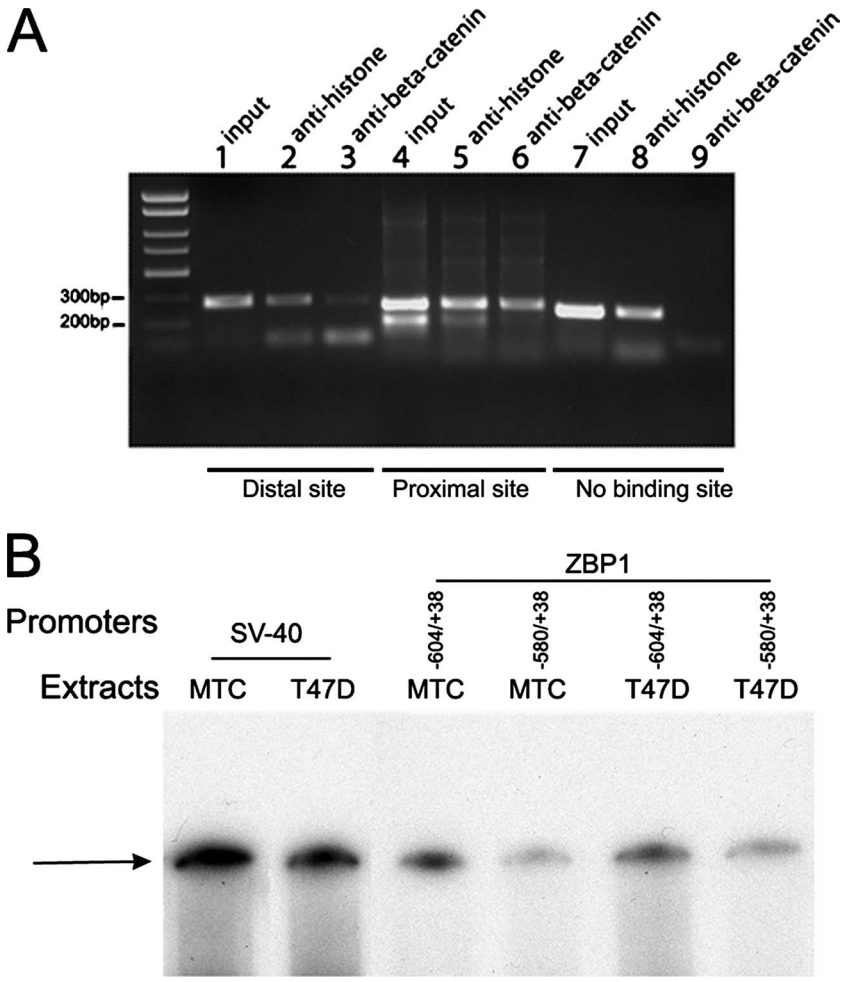

FIG. 5. The proximal binding site of the ZBP1 promoter physically associates with β-catenin in vivo and is required for transcription activity in vitro. (A) MTC cells were subjected to ChIP using antibodies against acetylated histone and β-catenin. Of the three primer pairs used, one amplified the region of the ZBP1 promoter containing the distal β-catenin binding site (lanes 1 to 3) and another amplified the proximal β-catenin binding site (lanes 4 to 6). The third primer pair was used to amplify the 5′ flanking region, which was approximately 2 kb upstream of the initiation site (lanes 7 to 9). β-Catenin antibody specifically pulled down the ZBP1 promoter containing the putative β-catenin binding sites (lanes 3 and 6) but not the chromatin region without the β-catenin/TCF4 recognition sequences (lane 9). (B) In vitro transcription of ZBP1 promoter reporters. Constructs under the control of the SV40 promoter and the ZBP1 promoters (−604 to +38 and −580 to +38) were incubated with crude nuclear extracts prepared from cultured MTC and T47D cells. The arrow points at the in vitro-transcribed runoff RNA products separated in a 6% urea gel.

sequences (lane 3). The β-catenin antibody did not precipitate the fragment without the putative binding element (lane 9). These results indicate a physical association of β-catenin with the CTTTG-TC element in the ZBP1 promoter.

The transcriptional activities of the ZBP1 promoter with or without the proximal β-catenin binding site were further examined in vitro using nuclear extracts prepared from cultured MTC and T47D cells. We employed three luciferase reporters as DNA templates for the assays: one was under the control of a viral SV40 promoter, and the other two reporters were driven by ZBP1 promoters (−604 to +38 and −585 to +38, respectively; Fig. 2C). The reporters, after predigestion with a restriction enzyme (BsrGI) which cut at the middle of the luciferase gene, would transcribe a runoff transcript of about 570 bp in the presence of nuclear extracts, In vitro transcription assays displayed that both nuclear extracts were able to efficiently transcribe the SV40 promoter-directed template. Transcribing the templates driven by the two different ZBP1 promoters produced different results. The relative in vitro transcriptional activity of the promoter that deleted the proximal binding site (−585 to +38) was reduced by nearly 70% (MTC) or 50% (T47D) relative to the promoter containing the β-catenin/TCF4 binding site (−604 to +38) (Fig. 5B). These in vitro transcription data were consistent with the in vivo data that effective transcription of the ZBP1 promoter required the proximal binding site.

β-Catenin binds the proximal CTTTG-TC element of the ZBP1 promoter in vitro. We performed the following biochemical experiments to test the in vitro interactions of β-catenin with its putative binding element in the ZBP1 promoter. First, we employed DNA mobility shift assays using a DNA oligonucleotide corresponding to the ZBP1 promoter region of −578 to −602, which contained the proximal CTTTG-TC sequence. We chose this element because it was associated with

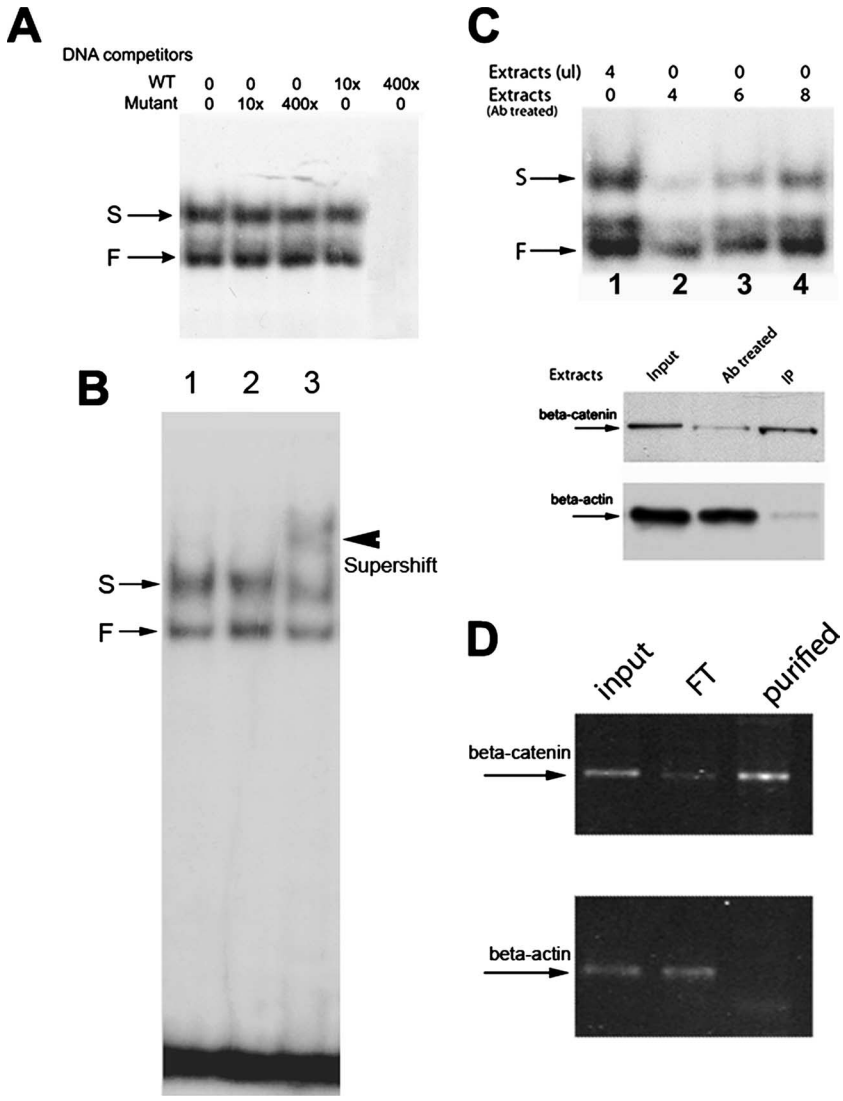

FIG. 6. β-Catenin binds to the ZBP1 promoter via the proximal binding site. (A) Nuclear extracts prepared from cultured MTC cells were incubated with a radiolabeled DNA fragment containing the proximal binding site (TCCCG-TC). Two DNA-protein complexes corresponding to DNA-TCF4 (F) and DNA-TCF4/β-catenin (S) were formed. The complexes were competed by unlabeled wild-type fragments (indicated as 0, 10×, and 400×) but not the equivalent mutant fragments. (B) Nuclear extracts of MTC cells were incubated with a radiolabeled TCCCG-TC-containing DNA fragment of the ZBP1 promoter in the absence of antibodies (lane 1) and in the presence of a nonspecific antibody (lane 2) or an antibody against β-catenin (lane 3). An arrowhead indicates the position of a supershift formed by the complex S with the β-catenin antibody. (C) β-catenin was partially immunoprecipitated from the nuclear extracts of MTC cells with a mouse antibody against the protein. The efficiency of immunoprecipitation was evaluated by Western blotting (lower half). β-Catenin but not β-actin was immunodepleted from the extracts. Different amounts of the β-catenin-depleted extracts (antibody [Ab]-treated extracts) were then used for DNA gel shift assays (upper half). The ability of the extracts to form both S and F complexes was greatly decreased but could be overcome by additional extract. (D) A biotin-labeled DNA fragment containing the putative proximal β-catenin/TCF4 binding site (−602 to −578) was bound to streptavidin beads and incubated with the nuclear extracts of MTC cells. After extensive washes, associated proteins were eluted and analyzed by Western blotting. β-Catenin but not β-actin was purified by the DNA fragment. Input, the total sample; FT, flowthrough; purified, eluted from the DNA element.

β-catenin in vivo and was essential for ZBP1 promoter activity. In addition, this element and its surrounding sequences were highly conserved in rat, mouse, and human cells. Incubation of the ^{32}P-labeled DNA fragment with nuclear extracts prepared from cultured MTC cells formed two DNA-protein complexes (Fig. 6A, complexes F and S). Presumably, the rapidly migrating band corresponded to the DNA-TCF4 complex and the slower-migrating band was the complex of DNA-TCF4/β-catenin (23). The specificity of the DNA-protein complexes was determined using competition assays, which demonstrated that in the presence of 400-fold excess of unlabeled specific DNA elements, binding of the complexes was completely competed. Formation of the complexes was not disrupted when up to 400-fold excess unlabeled mutant element (CTTTG to CT TCG) was used (Fig. 6A, middle lane). Second, we performed DNA mobility shift experiments in the presence of β-catenin antibody. Formation of the S and F complexes were not affected in the presence of a nonspecific mouse antibody (Fig.

6B, lane 2), while an additional slower migration complex was observed when a monoclonal antibody against β-catenin was present (Fig. 6B, lane 3), indicating that the β-catenin antibody was able to supershift the S complex. Third, we immunodepleted β-catenin from nuclear extracts with the antibody against the protein and incubated the extracts with the DNA element to identify whether complex formation required β-catenin. Western blot analysis indicated that β-catenin was largely diminished from the extracts after incubation with the antibody (Fig. 5C, lower panel). Depletion of β-catenin reduced the ability of the extracts to form the slower-moving complex to 13% (Fig. 6C, upper panel, comparison of lanes 1 and 2). Formation of the faster-migrating complex was also affected, possibly resulting from codepletion of TCF4 by β-catenin antibodies. However, the ability to form the complexes could be overcome with addition of extract (lanes 3 and 4). These results suggested that the formation of the specific DNA-protein complexes was β-catenin mediated. Finally, we applied an affinity pull-down assay using the CTTTG-TC-containing DNA fragment as a ligand (−602 to −578). The ligand was biotin labeled and attached to streptavidin beads that were incubated with nuclear extracts of cultured MTC cells. After extensive washing, associated proteins were eluted and analyzed by Western blotting using antibodies against β-catenin and β-actin (Fig. 6D). β-Catenin, but not β-actin, was specifically associated with the DNA ligand. β-Catenin was not precipitated with a control fragment where the CTTTG-TC element was mutated to CTTCG-TC (data not shown). These data further demonstrate the specific interaction of β-catenin with the ZBP1 promoter.

ZBP1 binds to β-catenin mRNA. ZBP1 expression directly affects cancer cell growth and metastasis (26, 28, 48). In order to identify the pathway regulated by ZBP1 that leads to the phenotypic changes, we immunoprecipitated ZBP1-containing mRNP particles from extracts of MTLn3 cells that did not express ZBP1 and a stably transformed MTLn3 cell line (MTLn3-ZBP1) that constitutively expressed ZBP1 (26). Extracted immunoprecipitated RNA samples were identified using a microarray analysis with Affymetrix rat 230 Plus 2.0 arrays, which represent over 28,000 well-substantiated mRNA transcripts. (Assays were performed at the Center for Functional Genomics at the University of Albany.) In two independent array screens, 890 transcripts (3.2% of expressed messages) were at least fivefold enriched in the MTLn3-ZBP1-IP mRNAs compared to the MTLn3-IP mRNAs (not shown). As expected, β-actin mRNA was identified as a major target of ZBP1 with a high binding affinity to the protein. Surprisingly, β-catenin mRNA also appeared in the list with an enrichment of 26.5-fold. The coding region of β-catenin mRNA contains two conserved ACACC motifs identified as required for ZBP1 binding (8). This suggested that ZBP1 could bind to β-catenin mRNA and play a role in feedback regulation of the transcript. To confirm whether β-catenin mRNA was physically associated with ZBP1 in vivo, we used ZBP1 antibodies to immunoprecipitate ZBP1-associated mRNAs in MTC extracts and tested for the presence of β-catenin mRNA by RT-PCR. We employed α-tubulin mRNA as a negative control and β-actin mRNA as a positive control, because it was well known to associate with ZBP1. A rabbit antiserum against ZBP1 could effectively precipitate both β-actin and β-catenin mRNAs but not α-tubulin mRNA (Fig. 7A, lanes 2, 3, and 4, respectively). A control RT-PCR experiment showed that the three mRNAs were detected in the cell extracts (Fig. 7A, lanes 5, 6, and 7, respectively). β-Catenin mRNA was not detected in the precipitates of a preimmune rabbit serum (Fig. 7A, lane 1), indicating that the cytoplasmic binding of ZBP1 to β-catenin mRNA was specific.

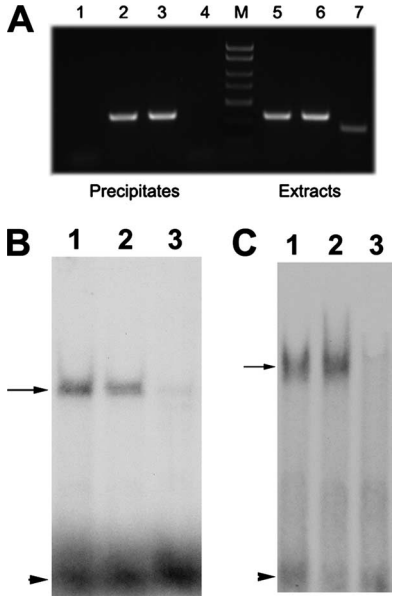

FIG. 7. ZBP1 binds to β-catenin mRNA in vivo and in vitro. (A) ZBP1 associates with cytoplamic β-catenin mRNA in breast cancer cells. Rabbit anti-ZBP1 serum was used to immunoprecipitate an MTC extract (Left). RT-PCR was used to detect the presence of β-catenin (lanes 2 and 5), β-actin (lanes 3 and 6), and α-tubulin (lanes 4 and 7) mRNAs in the precipitates and in total cell extracts (right). Lane 1 is an RT-PCR control to detect the presence of β-actin mRNA after an immunoprecipitation with rabbit preimmune serum. RNA gel shift assays used recombinant ZBP1 incubated with a ^{32}P-labeled RNA fragment located in the coding region of β-actin mRNA (+1583 to +1712) containing the ACACC motif (B) and with the ^{32}P-labeled 3′ UTR of β-catenin mRNA, respectively (C). The RNA-protein complexes formed were separated by electrophoresis in a 4% native gel and visualized by autoradiography (lanes 1). The specificity of ZBP1-RNA interactions was determined by competition assays, in which the corresponding unlabeled RNA fragment effectively abolished complex formation (lanes 3), while the unlabeled nonspecific RNA fragment of GAPDH mRNA did not compete the complexes (lanes 2). The positions of ZBP1-RNA complexes are indicated by arrows, and the free probes are indicated by arrowheads.

To determine whether ZBP1 directly binds to β-catenin mRNA, we performed gel shift assays using a recombinant ZBP1 that has been demonstrated to bind to the zipcode segment with high affinity (33). We in vitro synthesized three ^{32}P-labeled fragments of β-catenin mRNA. Two located in the coding region (+118 to +232 and +1583 to +1712 from the translational initiation site, respectively) each contained an ACACC motif. The third fragment was the 3′ UTR. When recombinant ZBP1 was incubated with the ^{32}P-labeled fragments, we did not observe the binding of the RNA fragment from +118 to +232 with ZBP1 in a mobility shift assay (not shown). However, distinct RNA-protein complexes were observed when ZBP1 was incubated with the fragment from +1583 to +1712 and the 3′ UTR of β-catenin mRNA (Fig. 7B

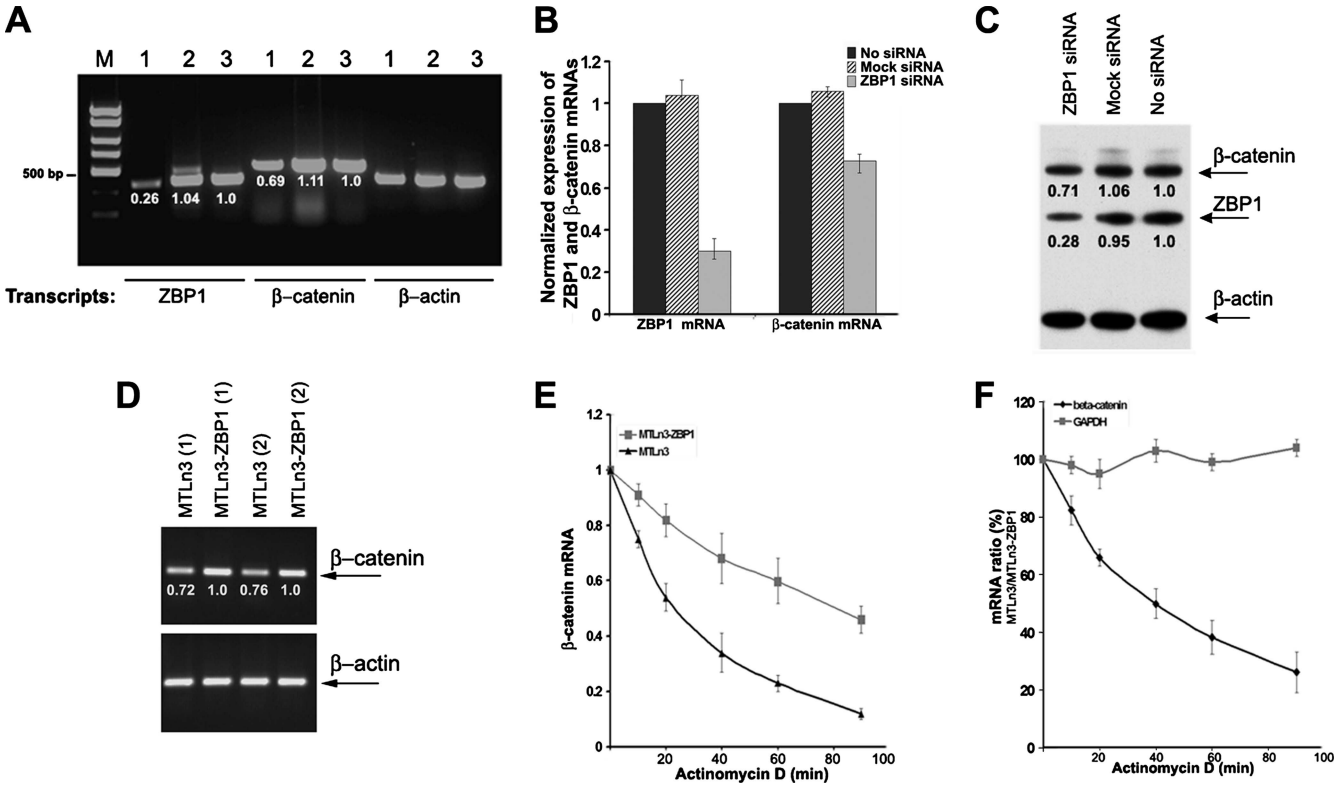

FIG. 8. ZBP1 expression increases the stability of β-catenin mRNA. (A) MTC cells were transfected with ZBP1 siRNA for 48 h and harvested. A representative RT-PCR shows the expression of β-catenin, ZBP1, and β-actin transcripts in transfected cells. Lane 1, ZBP1 siRNA-transfected cells; lane 2, scrambled-siRNA-transfected cells; and lane 3, no-siRNA-transfected cells. Numbers below the bands reflect reduction (*n*-fold) of ZBP1 and β-catenin mRNAs. (B) Relative levels of ZBP1 and β-catenin were normalized and averaged to β-actin from three independent experiments and plotted. (C) Immunoblot analysis of proteins isolated from siRNA-transfected cells as indicated. Numbers below the bands indicate relative levels of ZBP1 and β-catenin which were normalized to β-actin. The arrows indicate the detected proteins. (D) ZBP1 expression increases the abundance of β-catenin mRNA. Two independent RT-PCR experiments (1 and 2) were used to detect β-catenin and β-actin mRNAs in MTLn3 and MTLn3-ZBP1 cells. Numbers below the bands reflect relative levels of β-catenin mRNAs normalized to β-actin mRNA. The arrows indicate the detected transcripts. (E) Time course decay of β-catenin mRNA in MTLn3 and MTLn3-ZBP1 cells after treatment with actinomycin D. Relative mRNA ratios were determined by normalized to corresponding α-tubulin mRNA levels. (F) β-Catenin mRNA is selectively destabilized in ZBP1-negative MTLn3 cells. β-Catenin and GAPDH mRNA levels were analyzed by RT-PCR, and the relative mRNA ratios between parental MTLn3 and MTLn3-ZBP1 cells were determined.

and 7C, lanes 1). The specificity of the ZBP1-RNA complexes was determined by competition assays. Both complexes formed with the fragment from +1583 to +1712 and the 3′ UTR could be effectively competed with a 200× excess of corresponding unlabeled RNAs (Fig. 7B and C, lanes 3). In contrast, a fragment of nonspecific GAPDH RNA only slightly affected the complex formation (Fig. 7B and C, lanes 2). These experiments indicate that ZBP1 binds to β-catenin mRNA in vitro and one of the fragments contained the ACACC motif.

ZBP1 expression increases the stability of β-catenin mRNA. To determine the biological role of the ZBP1 and β-catenin mRNA interaction, we targeted knockdown of ZBP1 function in MTC cells by siRNA treatment and examined β-catenin expression (18). Transfection of ZBP1 siRNA into MTC cells resulted in significant downregulation of ZBP1 mRNA to 26% of that of the control (Fig. 8A and 8B). As a consequence of ZBP1 knockdown, cellular levels of β-catenin mRNA were also decreased to 69% of the control level (Fig. 8A and B) after 48 h of transfection. In contrast, expression of β-actin mRNA was not affected. Consistent with the mRNA results, loss of ZBP1 function also reduced the protein levels of β-catenin to 71% (Fig. 8C). The role of ZBP1 in β-catenin expression was further tested in ZBP1-negative MTLn3 cells and in stable MTLn3-ZBP1 cells constitutively expressing ZBP1 (24). ZBP1 reexpression in MTLn3 cells markedly increased the levels of β-catenin mRNA by nearly 28% (Fig. 8D). To determine that the β-catenin mRNA level was independent of transcription, the turnover of β-catenin mRNA was analyzed after blocking transcription by actinomycin D. In contrast to parental MTLn3 cells, β-catenin mRNA decay was significantly reduced in MTLn3-ZBP1 cells (Fig. 8E). Variations in cellular β-catenin mRNA and a non-ZBP1-associated mRNA (GAPDH) were also analyzed upon ZBP1 expression. β-Catenin mRNA was selectively destabilized in ZBP1-negative MTLn3 cells, whereas the stability of α-tubulin mRNA remained unaffected (Fig. 8F). These data suggest that binding of ZBP1 to β-catenin mRNA decreases its decay rate.

DISCUSSION

The ZBP1 family has the ability to regulate the expression of a plethora of RNA targets in order to link the processes of subcellular RNA localization, translational control, and RNA stability with cell polarity, migration, and proliferation (3, 26, 48). ZBP1, which is actively expressed during embryonic development, be-

comes undetectable after birth and is silenced in most adult tissues. However, activation or elevated expression of the gene has been detected in the vast majority of mammalian tumors of different origins, and in some tumor types, ZBP1 activation has become a characteristic marker of the samples studied (10, 17, 18, 35). A number of studies have revealed the association of ZBP1 expression with tumorigenesis and progression, including induction of mammary tumors (41), repression of cancer cell proliferation (26), and inhibition of metastasis (45). While much work has implicated the roles of ZBP1 in development and differentiation of human cancers (16, 20, 26), the mechanism responsible for gene activation has not been extensively studied. Understanding the mechanism could eventually reveal the underlying basis leading to the characteristic expression of the ZBP1 gene in human cancers and may even provide therapeutic implications for inhibiting carcinogenesis and metastasis.

In 293T cells, CRD-BP, a mouse ortholog of chicken ZBP1, could be activated in response to β-catenin signaling (31). In the present study, we confirmed this regulation and provided detailed evidence of molecular interaction between β-catenin and the ZBP1 promoter, which resulted in transactivation of the gene. We demonstrated that ZBP1 expression in mammalian breast cancer cells was correlated with nuclear translocation of endogenous β-catenin. We functionally characterized the ZBP1/CRD-BP promoter and identified an element (CTTTG-TC) located between nt −590 and −584 that was necessary for regulating gene expression. We further determined that β-catenin physically associated with the ZBP1 promoter through this putative binding element in vivo and specifically bound to the element in vitro. Noubissi et al. (31) identified that β-catenin expressed from a transfected plasmid bound to the region −1333 to −1607 of the human ZBP1/CRD-BP promoter (corresponding to the rat promoter of −1107 to −1381 upstream of the translation site). However, it is worthwhile to mention that this region is not necessary for promoter activity because reporters under the control of the ZBP1/CRD-BP promoter with or without it did not change their transcriptional activity. In comparison, the promoter region containing the CTTTG-TC element in the region from −590 to −584 we characterized for β-catenin binding is essential for the ZBP1/CRD-BP promoter activity and is highly conserved in rat, mouse, and human genes (as is its flanking region), suggesting that mammalian breast cancers use this conserved element to control ZBP1 transcription.

β-Catenin plays a critical role in cell morphogenesis and human carcinogenesis through its dual function as a component of the adhesive complex at cell-cell junctions and as a transcriptional coregulator in the *Wnt* signaling pathway (13). Normal cells contain low levels of β-catenin, mostly associated with cell membranes, due to the presence of the adenomatous polyposis coli (APC) protein, which mediates the degradation of free cytoplasmic β-catenin (36). Almost all colon cancers have either mutated β-catenin or deleted APC that allows free β-catenin to escape from cytoplasmic degradation and be imported into cell nuclei, where it activates the downstream targets of *Wnt*/β-catenin signaling (28). In breast cancers, aberrant activation of the β-catenin pathway has also been identified to induce a number of genes, including the cyclin D1, c-*myc*, Twist, vimentin, and E-cadherin genes. Most of these targets were involved in various physiological and pathological processes of the disease (9, 13, 15, 27, 39). We demonstrated that the ZBP1 gene is a target of the β-catenin pathway in breast cancers based on the in vivo and in vitro molecular interaction of β-catenin with the conserved element of the ZBP1 promoter, which had not been identified previously. Moreover, we identified β-catenin mRNA as a novel ZBP1 target. Gain of ZBP1 function increased stability of cytoplasmic β-catenin mRNA; apparently, this upregulation was contributed by the binding of ZBP1 to β-catenin mRNA. Thus, ZBP1 and β-catenin can regulate each other's expression, and this coregulation may open up new insights relating ZBP1 activation to breast cancers.

Both ZBP1 and β-catenin are well known to be highly expressed during normal embryonic development, as well as activated in a variety of human tumors (18, 25, 35). The oncofetal pattern of ZBP1 expression could be the result of β-catenin activation during embryogenesis and tumorigenesis. However, the feedback regulation of ZBP1 to β-catenin mRNA could potentially amplify the role of β-catenin in the *Wnt* signaling pathway to activate the genes associated with the pathway or to facilitate the function of β-catenin in cell-cell contacts. Targeted expression of ZBP1 in mammary tissues has been reported to induce mammary tumors, but this reduction was not by stabilizing c-*myc* mRNA (41). Therefore, tumor induction in the animal could be partially contributed by ZBP1 regulation to other mRNA targets, including β-catenin mRNA. In addition to the ability of ZBP1 to induce tumorigenesis, overexpression of ZBP1 in a K562 line inhibited cell proliferation (26); breast cancer cells without ZBP1 expression increased their metastatic potential (45). Given the roles of ZBP1 as a localizing factor and a translational regulator, it is likely that the contradictory effects of ZBP1 expression on breast cancer cells could result from the diversity of the mRNA targets of ZBP1—some of which inhibit growth and others of which stimulate motility. For instance, depending on the cell type from which the tumor is derived, ZBP1 could either promote cancer progression by controlling c-*myc* or β-catenin expression or repress metastasis by regulating localized translation of β-actin and other mRNAs.

In summary, we report an interconnected regulatory mechanism that leads to the characteristic expression of ZBP1 and β-catenin in breast cancer cells. β-Catenin binds to the conserved element of the mammalian ZBP1 promoter and transactivates gene expression, while ZBP1 associates with β-catenin mRNA and controls its cytoplasmic expression. This is the first demonstration that these two gene products are coregulated. The loss of function in either gene will affect the other's expression. The strong correlation between β-catenin signaling and ZBP1 activation is found in both breast cancer cell lines and breast patient tumors. Future studies will define the physiological significance of this interrelationship.

ACKNOWLEDGMENTS

This study was supported by NIH grants CA83208 and AR41480 to R.H.S.

We thank K. Lapidus for kindly providing the MTLn3-ZBP1 stable cell line and members of the lab for valuable discussions and suggestions.

REFERENCES

1. **Capodieci, P., M. Donovan, H. Buchinsky, Y. Jeffers, C. Cordon-Cardo, W. Gerald, J. Edelson, S. M. Shenoy, and R. H. Singer.** 2005. Gene expression profiling in single cells within tissue. Nat. Methods **2:**663–665.

2. **Castrop, J., K. van Norren, and H. Clevers.** 1992. A gene family of HMG-box transcription factors with homology to TCF-1. Nucleic Acids Res. **20:**611.
3. **Condeelis, J., and R. H. Singer.** 2005. How and why does beta-actin mRNA target? Biol. Cell **97:**97–110.
4. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
5. **Doyle, G. A., N. A. Betz, P. F. Leeds, A. J. Fleisig, R. D. Prokipcak, and J. Ross.** 1998. The c-myc coding region determinant-binding protein: a member of a family of KH domain RNA-binding proteins. Nucleic Acids Res. **26:** 5036–5044.
6. **Elbashir, S. M., J. Harborth, K. Weber, and T. Tuschl.** 2002. Analysis of gene function in somatic mammalian cells using small interfering RNAs. Methods **26:**199–213.
7. **Elbashir, S. M., W. Lendeckel, and T. Tuschl.** 2001. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15:**188–200.
8. **Gavert, N., M. Conacci-Sorrell, D. Gast, A. Schneider, P. Altevogt, T. Brabletz, and A. Ben-Ze'ev.** 2005. L1, a novel target of beta-catenin signaling, transforms cells and is expressed at the invasive front of colon cancers. J. Cell Biol. **168:**633–642.
9. **Gilles, C., M. Polette, M. Mestdagt, B. Nawrocki-Raby, P. Ruggeri, P. Birembaut, and J. M. Foidart.** 2003. Transactivation of vimentin by beta-catenin in human breast cancer cells. Cancer Res. **63:**2658–2664.
10. **Gu, L., K. Shigemasa, and K. Ohama.** 2004. Increased expression of IGF II mRNA-binding protein 1 mRNA is associated with an advanced clinical stage and poor prognosis in patients with ovarian cancer. Int. J. Oncol. **24:**671–678.
11. **Gu, W., C. Morales, and N. B. Hecht.** 1995. In male mouse germ cells, copper-zinc superoxide dismutase utilizes alternative promoters that produce multiple transcripts with different translation potential. J. Biol. Chem. **270:**236–243.
12. **Hansen, T. V. O., N. A. Hammer, J. Nielsen, M. Madsen, C. Dalbaeck, U. M. Wewer, J. Christiansen, and F. C. Nielsen.** 2004. Dwarfism and impaired gut development in insulin-like growth factor II mRNA-binding protein 1-deficient mice. Mol. Cell. Biol. **24:**4448–4464.
13. **Hatsell, S., T. Rowlands, M. Hiremath, and P. Cowin.** 2003. Beta-catenin and Tcfs in mammary development and cancer. J. Mammary Gland Biol. Neoplasia **8:**145–158.
14. **Hovanes, K., T. W. Li, and M. L. Waterman.** 2000. The human LEF-1 gene contains a promoter preferentially active in lymphocytes and encodes multiple isoforms derived from alternative splicing. Nucleic Acids Res. **28:**1994–2003.
15. **Howe, L. R., O. Watanabe, J. Leonard, and A. M. Brown.** 2003. Twist is up-regulated in response to Wnt1 and inhibits mouse mammary cell differentiation. Cancer Res. **63:**1906–1913.
16. **Huttelmaier, S., D. Zenklusen, M. Lederer, J. Dictenberg, M. Lorenz, X. Meng, G. J. Bassell, J. Condeelis, and R. H. Singer.** 2005. Spatial regulation of beta-actin translation by Src-dependent phosphorylation of ZBP1. Nature **438:**512–515.
17. **Ioannidis, P., L. Mahaira, A. Papadopoulou, M. R. Teixeira, S. Heim, J. A. Andersen, E. Evangelou, U. Dafni, N. Pandis, and T. Trangas.** 2003. 8q24 copy number gains and expression of the c-myc mRNA stabilizing protein CRD-BP in primary breast carcinomas. Int. J. Cancer **104:**54–59.
18. **Ioannidis, P., L. Mahaira, A. Papadopoulou, M. R. Teixeira, S. Heim, J. A. Andersen, E. Evangelou, U. Dafni, N. Pandis, and T. Trangas.** 2003. CRD-BP: a c-Myc mRNA stabilizing protein with an oncofetal pattern of expression. Anticancer Res. **23:**2179–2183.
19. **Ioannidis, P., L. G. Mahaira, S. A. Perez, A. D. Gritzapis, P. A. Sotiropoulou, G. J. Kavalakis, A. I. Antsaklis, C. N. Baxevanis, and M. Papamichail.** 2005. CRD-BP/IMP1 expression characterizes cord blood CD34+ stem cells and affects c-myc and IGF-II expression in MCF-7 cancer cells. J. Biol. Chem. **280:**20086–20093.
20. **Ioannidis, P., T. Trangas, E. Dimitriadis, M. Samiotaki, I. Kyriazoglou, C. M. Tsiapalis, C. Kittas, N. Agnantis, F. C. Nielsen, J. Nielsen, J. Christiansen, and N. Pandis.** 2001. C-MYC and IGF-II mRNA-binding protein (CRD-BP/IMP-1) in benign and malignant mesenchymal tumors. Int. J. Cancer **94:**480–484.
21. **Kislauskis, E. H., X. Zhu, and R. H. Singer.** 1997. β-Actin messenger RNA localization and protein synthesis augment cell motility. J. Cell Biol. **136:** 1263–1270.
22. **Kobel, M., D. Weidensdorfer, C. Reinke, M. Lederer, W. D. Schmitt, K. Zeng, C. Thomssen, S. Hauptmann, and S. Huttelmaier.** 2007. Expression of the RNA-binding protein IMP1 correlates with poor prognosis in ovarian carcinoma. Oncogene **26:**7584–7589.
23. **Korinek, V., N. Barker, P. J. Morin, D. van Wichen, R. de Weger, K. W. Kinzler, B. Vogelstein, and H. Clevers.** 1997. Constitutive transcriptional activation by a β-catenin-Tcf complex in $APC^{-/-}$ colon carcinoma. Science **275:**1784–1787.
24. **Lapidus, K., J. Wyckoff, G. Mouneimne, M. Lorenz, L. Soon, J. S. Condeelis, and R. H. Singer.** 2007. ZBP1 enhances cell polarity and reduces chemotaxis. J. Cell Sci. **120:**3173–3178.
25. **Leeds, P., B. T. Kren, J. M. Boylan, N. A. Betz, C. J. Steer, P. A. Gruppuso, and J. Ross.** 1997. Developmental regulation of CRD-BP, an RNA-binding protein that stabilizes c-myc mRNA in vitro. Oncogene **14:**1279–1286.
26. **Liao, B., M. Patel, Y. Hu, S. Charles, D. J. Herrick, and G. Brewer.** 2004. Targeted knockdown of the RNA-binding protein CRD-BP promotes cell proliferation via an insulin-like growth factor II-dependent pathway in human K562 leukemia cells. J. Biol. Chem. **279:**48716–48724.
27. **Lin, S. Y., W. Xia, J. C. Wang, K. Y. Kwong, B. Spohn, Y. Wen, R. G. Pestell, and M. C. Hung.** 2000. Beta-catenin, a novel prognostic marker for breast cancer: its roles in cyclin D1 expression and cancer progression. Proc. Natl. Acad. Sci. USA **97:**4262–4266.
28. **Logan, C. Y., and R. Nusse.** 2004. The Wnt signaling pathway in development and disease. Annu. Rev. Cell Dev. Biol. **20:**781–810.
29. **Nielsen, F. C., J. Nielsen, and J. Christiansen.** 2001. A family of IGF-II mRNA binding proteins (IMP) involved in RNA trafficking. Scand. J. Clin. Lab. Invest. Suppl. **234:**93–99.
30. **Nielsen, J., J. Christiansen, J. Lykke-Andersen, A. H. Johnsen, U. M. Wewer, and F. C. Nielsen.** 1999. A family of insulin-like growth factor II mRNA-binding proteins represses translation in late development. Mol. Cell. Biol. **19:**1262–1270.
31. **Noubissi, F. K., I. Elcheva, N. Bhatia, A. Shakoori, A. Ougolkov, J. Liu, T. Minamoto, J. Ross, S. Y. Fuchs, and V. S. Spiegelman.** 2006. CRD-BP mediates stabilization of betaTrCP1 and c-myc mRNA in response to beta-catenin signalling. Nature **441:**898–901.

31a. **Oleynikov, Y.** 1999. Ph.D. thesis. Albert Einstein College of Medicine, Bronx, NY.

32. **Oleynikov, Y., and R. H. Singer.** 2003. Real-time visualization of ZBP1 association with beta-actin mRNA during transcription and localization. Curr. Biol. **13:**199–207.
33. **Pan, F., S. Hüttelmaier, R. H. Singer, and W. Gu.** 2007. ZBP2 facilitates binding of ZBP1 to β-actin mRNA during transcription. Mol. Cell. Biol. **27:**8340–8351.
34. **Ross, A. F., Y. Oleynikov, E. H. Kislauskis, K. L. Taneja, and R. H. Singer.** 1997. Characterization of a β-actin mRNA zipcode-binding protein. Mol. Cell. Biol. **17:**2158–2165.
35. **Ross, J., I. Lemm, and B. Berberet.** 2001. Overexpression of an mRNA-binding protein in human colorectal cancer. Oncogene **20:**6544–6550.
36. **Rubinfeld, B., I. Albert, E. Porfiri, C. Fiol, S. Munemitsu, and P. Polakis.** 1996. Binding of GSK3beta to the APC-beta-catenin complex and regulation of complex assembly. Science **272:**1023–1026.
37. **Runge, S., F. C. Nielsen, J. Nielsen, J. Lykke-Andersen, U. M. Wewer, and J. Christiansen.** 2000. H19 RNA binds four molecules of insulin-like growth factor II mRNA-binding protein. J. Biol. Chem. **275:**29562–29569.
38. **Sawadogo, M., and R. G. Roeder.** 1985. Factors involved in specific transcription by human RNA polymerase II: analysis by a rapid and quantitative in vitro assay. Proc. Natl. Acad. Sci. USA **82:**4394–4398.
39. **Sinn, E., W. Muller, P. Pattengale, I. Tepler, R. Wallace, and P. Leder.** 1987. Coexpression of MMTV/v-Ha-ras and MMTV/c-myc genes in transgenic mice: synergistic action of oncogenes in vivo. Cell **49:**465–475.
40. **Stadeli, R., R. Hoffmans, and K. Basler.** 2006. Transcription under the control of nuclear Arm/beta-catenin. Curr. Biol. **16:**R378–R385.
41. **Tessier, C. R., G. A. Doyle, B. A. Clark, H. C. Pitot, and J. Ross.** 2004. Mammary tumor induction in transgenic mice expressing an RNA-binding protein. Cancer Res. **64:**209–214.
42. **Travis, A., A. Amsterdam, C. Belanger, and R. Grosschedl.** 1991. LEF-1, a gene encoding a lymphoid-specific protein with an HMG domain, regulates T-cell receptor alpha enhancer function. Genes Dev. **5:**880–894.
43. **van de Wetering, M., M. Oosterwegel, D. Dooijes, and H. Clevers.** 1991. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. EMBO J. **10:**123–132.
44. **Vikesaa, J., T. V. Hansen, L. Jonson, R. Borup, U. M. Wewer, J. Christiansen, and F. C. Nielsen.** 2006. RNA-binding IMPs promote cell adhesion and invadopodia formation. EMBO J. **25:**1456–1468.
45. **Wang, W., S. Goswami, K. Lapidus, A. L. Wells, J. B. Wyckoff, E. Sahai, R. H. Singer, J. E. Segall, and J. S. Condeelis.** 2004. Identification and testing of a gene expression signature of invasive carcinoma cells within primary mammary tumors. Cancer Res. **64:**8585–8594.
46. **Wang, W., J. B. Wyckoff, V. C. Frohlich, Y. Oleynikov, S. Huttelmaier, J. Zavadil, L. Cermak, E. P. Bottinger, R. H. Singer, J. G. White, J. E. Segall, and J. S. Condeelis.** 2002. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. Cancer Res. **62:**6278–6288.
47. **Yaniv, K., and J. K. Yisraeli.** 2002. The involvement of a conserved family of RNA binding proteins in embryonic development and carcinogenesis. Gene **287:**49–54.
48. **Yisraeli, J. K.** 2005. VICKZ proteins: a multi-talented family of regulatory RNA-binding proteins. Biol. Cell **97:**87–96.
49. **Yiu, G. K., M. T. Murray, and N. B. Hecht.** 1997. Deoxyribonucleic acid-protein interactions associated with transcriptional initiation of the mouse testis-specific cytochrome c gene. Biol. Reprod. **56:**1439–1449.