CBP_c–mediated transcription but activated transcription mediated by the prototypical transcription factor target of the MAP kinase cascade, Elk-1 (*24, 25*). These results demonstrate that CBP is a nuclear Ca^{2+}/CaM kinase IV–regulated coactivator.

The identification of a nuclear Ca^{2+}/CaM kinase IV and cAMP-regulated transcriptional activation domain in CBP suggests a two-step model for CREB/CBP-mediated gene expression. In this model the CBP recruitment signal, which renders the CREB/CBP complex transcriptionally competent, has to coincide with a CBP activating signal to stimulate transcription. The mechanism by which nuclear Ca^{2+} and cAMP regulate CBP activity may involve phosphorylation of CBP. CBP is a phosphoprotein but we were unable to detect a change in the overall phosphorylation of CBP upon activation of Ca^{2+} signaling pathways, and mutations of putative CaM kinase phosphorylation sites in the COOH-terminal activation domain did not affect CBP-mediated transcriptional activation (*26*). Although CBP activity is differentially controlled by spatially distinct Ca^{2+} signals, CREB phosphorylation on Ser^{133} is a common end point for many signaling pathways. Thus, CREB functions as a sensor for cell activation and may, in the absence of CBP activating signals, serve a general supportive role in transcriptional responses that are primarily mediated by other activators such as Elk-1 or the serum response factor (*25, 27*). The importance of nuclear Ca^{2+} and cAMP in stimulating CBP activity may explain why growth factor receptor tyrosine kinases, which do not elicit Ca^{2+} transients or cause increases in the intracellular concentration of cAMP, are poor activators of CRE/CREB–dependent transcription (*25, 27, 28*), despite their ability to efficiently induce CREB phosphorylation on Ser^{133} via the MAP kinase pathway (*27, 29*). Given that many transcriptional activators can recruit CBP to a promoter, control of CBP function by nuclear Ca^{2+} and cAMP may prove to be of general importance for signal-regulated transcription.

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Identification of c-*MYC* as a Target of the APC Pathway

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The adenomatous polyposis coli gene (*APC*) is a tumor suppressor gene that is inactivated in most colorectal cancers. Mutations of *APC* cause aberrant accumulation of β-catenin, which then binds T cell factor–4 (Tcf-4), causing increased transcriptional activation of unknown genes. Here, the c-*MYC* oncogene is identified as a target gene in this signaling pathway. Expression of c-*MYC* was shown to be repressed by wild-type APC and activated by β-catenin, and these effects were mediated through Tcf-4 binding sites in the c-*MYC* promoter. These results provide a molecular framework for understanding the previously enigmatic overexpression of c-MYC in colorectal cancers.

Most human colorectal tumors are initiated by inactivation of the *APC* tumor suppressor gene, located on chromosome 5q21 (*1*). APC is a cytoplasmic protein that can bind to and promote the degradation of β-catenin (*2*). Among β-catenin functions is the ability to bind members of the Tcf family of transcription factors and activate gene transcription (*3*). Accordingly, human colorectal tumors with APC or β-catenin mutations

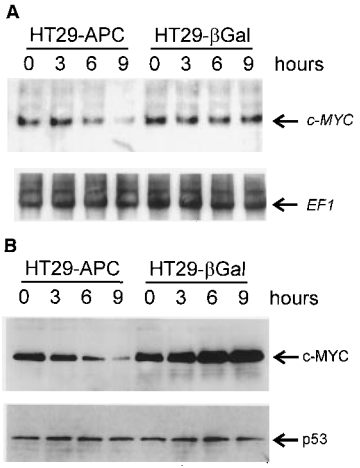

Fig. 1. c-*MYC* expression after APC induction. (**A**) Total RNA was isolated from the $ZnCl_2$-treated cells at the indicated times and evaluated on Northern blots (10 μg of RNA per lane) that were hybridized with a probe for c-MYC or a control probe for elongation factor 1α mRNA (*EF1*). (**B**) Total cellular proteins from the same cells were separated by SDS–polyacrylamide gel electrophoresis and subjected to immunoblotting with a monoclonal antibody to c-MYC (9E10, Santa Cruz Biotechnology). An identical blot probed with a monoclonal antibody to p53 shows that equal amounts of protein were loaded in each lane.

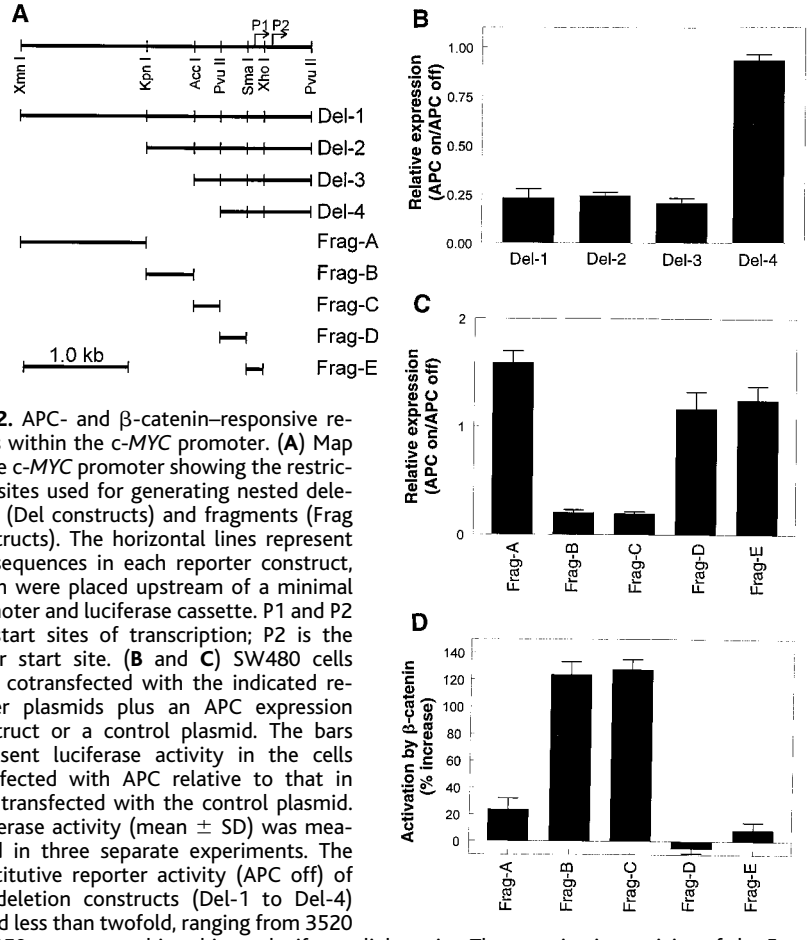

Fig. 2. APC- and β-catenin–responsive regions within the c-*MYC* promoter. (**A**) Map of the c-*MYC* promoter showing the restriction sites used for generating nested deletions (Del constructs) and fragments (Frag constructs). The horizontal lines represent the sequences in each reporter construct, which were placed upstream of a minimal promoter and luciferase cassette. P1 and P2 are start sites of transcription; P2 is the major start site. (**B** and **C**) SW480 cells were cotransfected with the indicated reporter plasmids plus an APC expression construct or a control plasmid. The bars represent luciferase activity in the cells transfected with APC relative to that in cells transfected with the control plasmid. Luciferase activity (mean ± SD) was measured in three separate experiments. The constitutive reporter activity (APC off) of the deletion constructs (Del-1 to Del-4) varied less than twofold, ranging from 3520 to 6859 as expressed in arbitrary luciferase light units. The constitutive activity of the Frag-A, -B, -C, -D, and -E was 364, 3050, 1063, 1754, and 976, respectively. (**D**) 293 cells were cotransfected with the indicated reporters plus a β-catenin expression construct or a control plasmid. The increase in luciferase activity in the β-catenin transfectants relative to the control transfectants is plotted on the *y* axis. Data are presented as the mean ± SD determined from three separate transfections.

exhibit increased β-catenin/Tcf–mediated transcription (*4*, *5*). However, the downstream targets of this β-catenin/Tcf-4–regulated transcription are unknown. This study was undertaken to define those targets and thereby gain clues to the mechanisms through which APC affects cellular growth.

To evaluate the transcriptional effects of APC, we studied a human colorectal cancer cell line (HT29-APC) containing a zinc-inducible *APC* gene and a control cell line (HT29–β-Gal) containing an analogous inducible *lacZ* gene (*6*). Both endogenous *APC* alleles in HT29 cells contain truncating mutations, and restoration of wild-type (WT) APC expression results in growth inhibition and apoptosis. Upon induction, APC protein is synthesized rapidly and reaches maximal levels by 9 hours (*7*). By 12 hours, a significant fraction of the cells display morphological signs of apoptosis. Because we were interested in identifying changes in gene expression that directly relate to restoration of APC function and not apoptosis, we analyzed the HT29-APC cells 9 hours after APC induction.

To evaluate changes in gene expression, we used serial analysis of gene expression (SAGE), a technique that allows the quantitative evaluation of cellular mRNA in an unbiased manner (*8*). In brief, the method is based on the use of short sequence tags [15 base pairs (bp)] generated from defined positions within each transcript. Expression levels are deduced from the abundance of individual tags in a sample. SAGE analysis of 51,622 and 55,846 tags from APC-induced and control cells, respectively, allowed identification of 14,346 different transcripts (*9*), most of which were expressed at similar levels in the APC-induced and control cells. Of the 30 tags showing significant differences in expression (*10*), 14 were overexpressed and 16 were repressed in APC-induced cells. Because biochemical studies have indicated that APC represses β-catenin/Tcf-4–mediated transcription (*4*, *5*), we focused on the latter transcripts. One of the three most highly repressed transcripts was a tag corresponding to the c-*MYC* oncogene (eight tags in HT29–β-Gal compared with zero in HT29-APC). This repression was confirmed at the mRNA and protein level by Northern (RNA) blot (Fig. 1A) and immunoblot (Fig. 1B) analysis, respectively. Repression of c-*MYC* mRNA and protein was evident within 6 hours after zinc induction and within 3 hours after the first detection of APC protein (Fig. 1).

These results suggested that APC might directly modulate c-*MYC* transcription through β-catenin/Tcf-4. To assess this possibility, we isolated a 2.5-kb genomic fragment encompassing the c-*MYC* promoter, inserted it upstream of a luciferase reporter gene (*11*), and then tested the construct for responsiveness to APC (*12*). This c-*MYC* promoter region conferred significant transcriptional activity to the basal reporter gene when transfected into human colorectal cancer cells, and this activity was significantly repressed by

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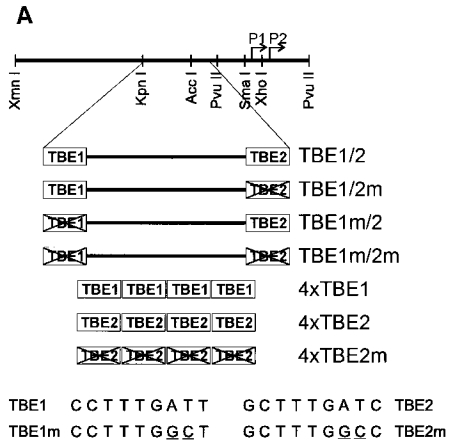

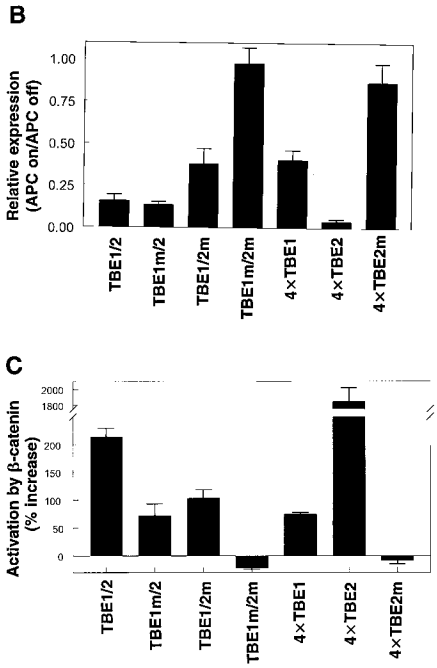

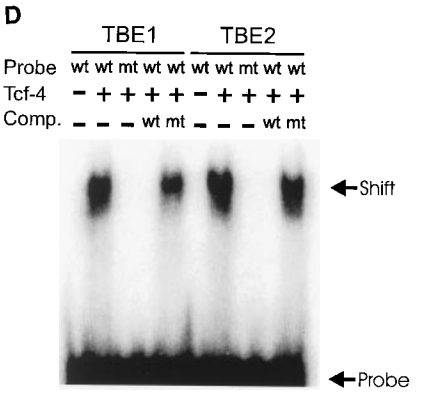

Fig. 3. Tcf-4 binding elements (TBEs) within the c-*MYC* promoter. (**A**) Map of the c-*MYC* promoter, indicating the 2.5-kb region containing the APC- and β-catenin–responsive elements. The fragment containing the WT sequence of the promoter (TBE1/2) contains TBE sites near both ends. This fragment was engineered to contain mutations in either site 1 (TBE1m/2), 2 (TBE1/2m), or both sites 1 and 2 (TBE1m/2m), and each fragment was placed upstream of a minimal promoter and luciferase reporter. Reporters containing four copies of TBE1 (4×TBE1) or TBE2 (4×TBE2) or a mutant TBE2 (4×TBE2m), in the absence of any additional genomic sequences, were constructed similarly. (**B**) SW480 cells were cotransfected with the indicated reporter plasmids plus an APC expression construct or a control plasmid. Data are presented as in Fig. 2B. (**C**) 293 cells were cotransfected with the indicated reporters plus a β-catenin expression construct or a control plasmid. The increase in luciferase activity in the β-catenin transfectants relative to the control transfectants is plotted on the *y* axis. Data are presented as the mean ± SD determined from three separate transfections. (**D**) Electrophoretic mobility shift assay. Oligonucleotides containing TBE1 or TBE2 sequences (wt) or mutants (mt) with nucleotide substitutions at critical positions were end-labeled with [γ-^{32}P]ATP and incubated with 0.5 μg of a GST–fusion protein containing the DNA-binding domain of Tcf-4. DNA-protein complexes were separated by electrophoresis and detected as "shifts" from the position of free probe. Unlabeled oligonucleotides (250 ng) were used as competitors (Comp.) in some reactions.

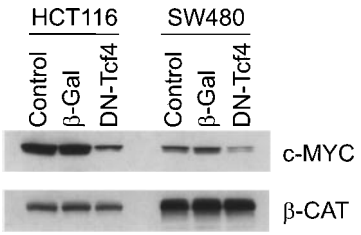

Fig. 4. Repression of c-MYC expression by a dominant-negative Tcf-4. Exponentially growing HCT116 and SW480 cells were mock-infected (Control) or infected with adenovirus encoding dominant-negative Tcf4 (DN-Tcf4) or β-galactosidase (β-Gal) at a multiplicity of infection of 100. Total cellular proteins were isolated 24 hours after infection and subjected to immunoblotting with a monoclonal antibody to c-MYC (C-19, Santa Cruz Biotechnology). An identical blot probed with a monoclonal antibody to β-catenin (Transduction Laboratories) shows that β-catenin is intact and that equal amounts of protein were loaded in each lane. The recombinant adenoviruses were constructed by means of the AdEasy system (*26*), the details of which are available upon request.

APC (Fig. 2). Nested deletions of the promoter were used to map the APC-responsive region to a fragment containing nucleotides (nt) −1194 to −484 relative to the TATA box at the c-*MYC* major transcription start site (Fig. 2, A and B). Testing of restriction fragments spanning the promoter revealed two responsive regions, one located in fragment B (nt −1194 to −741) and the other in fragment C (nt −741 to −484) (Fig. 3, A and C).

If the effects of APC on c-*MYC* transcription were mediated through inhibition of β-catenin/Tcf-4–regulated transcription, then the c-*MYC* promoter should be activated by β-catenin. Previous studies have shown that β-catenin/Tcf-4 transcription can be activated by exogenous expression of a mutant β-catenin gene in the human kidney cell line 293. The β-catenin construct used for these experiments was mutated at codon 33, rendering it insensitive to down-regulation by the endogenous WT APC in 293 cells (*5*). The c-*MYC* reporter was found to be significantly activated by β-catenin in this line. Using the nested deletion and restriction fragment constructs noted above, we found that the region of the c-*MYC* promoter that conferred β-catenin responsiveness was the same region (fragments B and C) shown to be APC-repressible in colorectal cancer cells (Fig. 2D).

Analysis of the c-*MYC* promoter sequence revealed one potential Tcf-4 binding site (*13*) within fragment B (TBE1) and another within fragment C (TBE2) (Fig. 3A). To test the functional significance of these sites, we created fragments of the c-*MYC* promoter in which one or both binding sites were eliminated by nucleotide substitutions (*14*). Mutation of either TBE1 or TBE2 reduced the activity of the c-*MYC* promoter fragment by 50%. Importantly, deletion of both sites completely removed APC repression and β-catenin activation from the reporter, whereas deletion of either element alone did not abrogate responsiveness (Fig. 3, A to C).

We also tested the TBE1 and TBE2 elements in isolation. Constructs containing four tandem copies of either TBE1 or TBE2 upstream of a minimal promoter (*15*) conferred β-catenin responsiveness and APC repression to a downstream luciferase reporter (Fig. 3, A to C). In all cases, the responsiveness of the reporter containing TBE2 sites was greater than that obtained with TBE1 sites. Nucleotide substitutions within TBE1 or TBE2 that would be expected to abolish Tcf-4 binding abrogated responsiveness to APC and β-catenin (Fig. 3, A to C). Finally, to confirm the direct nature of the responsiveness, we tested the ability of Tcf-4 produced in bacteria to bind TBE1 and TBE2 (*16*). Tcf-4 bound both TBE1 and TBE2, as judged by electrophoretic mobility-shift assay, and this binding was abrogated by the same nucleotide substitutions that eliminated transcriptional responses (Fig. 3D).

On the basis of these data, we propose that in normal colorectal epithelial cells, WT APC prevents β-catenin from forming a complex with Tcf-4 and activating c-*MYC*. In colorectal tumors with APC mutations or activating β-catenin mutations,

increased β-catenin/Tcf-4 activity leads to overexpression of c-*MYC*, which then promotes neoplastic growth. Consistent with this model, expression of a dominant-negative Tcf-4 in colorectal cancer cells with mutant β-catenin (HCT116) or mutant APC (SW480) significantly reduced the endogenous levels of c-MYC (Fig. 4). This model is also consistent with the powerful oncogenic activities of c-MYC (*17*) and provides an explanation for two long-standing quandaries. First, it has been extensively documented that c-*MYC* is overexpressed at the RNA and protein levels at both early and late stages of colorectal tumorigenesis (*18*). However, unlike in some other cancers, where the c-*MYC* gene is rearranged or amplified (*19*), genetic alterations of c-*MYC* are rare in colorectal tumors, and the cause of the overexpression has been unknown (*20*). The only clue to this mechanism has come from chromosome transfer experiments, in which it was shown that an extra copy of chromosome 5 can repress c-*MYC* transcription and inhibit neoplastic growth (*21*). This repression fits well with the molecular data presented here on *APC* which resides on chromosome 5q21.

The second enigma involves the cyclin-dependent kinase inhibitor p16INK4a. Most tumor types exhibit genetic alterations of the p16INK4a growth-inhibitory pathway through direct mutation of p16INK4a, its neighbor p15INK4b, or its downstream targets Rb, cdk4, or cyclin D1 (*22*). Colorectal cancers are a notable exception, in that few mutations of any of the genes in this pathway occur (*22*, *23*). The activation of c-*MYC* through APC inactivation would explain this, as c-MYC expression can bypass p16INK4a- and p15INK4b-mediated growth arrest (*24*).

Note added in proof: Consistent with a critical role for β-catenin/Tcf4–stimulated c-MYC expression in promoting intestinal cell proliferation, recent genetic studies in mice indicate that Tcf-4 is required to maintain the proliferative compartment in intestinal crypts (*25*).

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9. SAGE was performed as in (*8*) on mRNA from exponentially growing HT29-APC and HT29–β-Gal cells 9 hours after induction. A total of 55,233 and 59,752 tags were obtained from HT29-APC and HT29–β-Gal cells, respectively. Analysis of internal linker controls revealed a sequencing error rate of 0.065 per tag, corresponding to a sequencing error rate of 0.0067 per base. This was in good agreement with instrument specifications and previous estimates of SAGE tag errors based on analysis of the completed yeast genome (*8*). After correcting for sequencing mistakes, a total of 107,468 tags representing 51,622 and 55,846 from HT29-APC and HT29–β-Gal cells, respectively, were analyzed. These tags represented 14,346 unique transcripts, of which 7811 transcripts appeared at least twice.
10. Expression differences were considered significant if they had a P_{False} of <0.1 as determined by Monte Carlo simulations and they were at least fivefold in magnitude (*8*).
11. A low–basal activity reporter plasmid, pBV-Luc, was first constructed. The pDel-1, pDel-2, pDel-3, pDel-4, pFrag-A, pFrag-B, pFrag-C, pFrag-D, and pFrag-E reporters were constructed by cloning corresponding restriction fragments (illustrated in Fig. 2A) of human c-*MYC* promoter into pBV-Luc. Details of vector construction are available upon request.
12. Exponentially growing SW480 and 293 cells were cultured in 12-well plates and transfected with 0.4 μg of reporter, 0.2 μg of pCMVβGal control, and 0.9 μg of effector plasmid with LipofectAmine (Life Technologies). The APC [K. J. Smith *et al*., *Cancer Res*. **54**, 3672 (1994)] and β-catenin (*5*) effector plasmids have been described. Luciferase assays were carried out 24 hours after transfection and normalized for transfection efficiency through β-galactosidase activity. Each assay was performed in triplicate.
13. Two TBE-binding elements were identified in the region conferring APC and β-catenin responsiveness. TBE1 (CTTTGAT) was located 1156 bp upstream of the TATA box at the P1 transcription start site and perfectly matched the consensus for Tcf-binding CTTTG(A/T)(A/T) [M. van de Wetering, M. Oosterwegel, D. Dooijes, H. Clevers, *EMBO J.* **10**, 123 (1991); K. Giese, A. Amsterdam, R. Grosschedl, *Genes Dev*. **5**, 2567 (1991)]. TBE2 was located 589 bp upstream of the TATA box and contained an inverted perfect match (ATCAAAG). A third Tcf-binding site was located 1400 bp upstream of the TATA box but did not overlap with APC or β-catenin responsiveness.
14. To construct pTBE1/2 plasmid, we used polymerase chain reaction (PCR) primers (5′-CTAGCTAGCCTAGCACCTTTGATTTCTCCC-3′ and 5′-CGTGATATCCGCTTTGATCAAGAGTCCCAG-3′) to amplify nt −576 to −1162 of the c-*MYC* promoter region. The PCR product was cloned into pBV-Luc. To construct pTBE1/2m, pTBE1m/2, and pTBE1m/2m, we used a mutated TBE1 primer (5′-CTAGCTAGCACTGGTGCATCTCCCAAACCCGGCAGCCCG-3′) and a mutated TBE2 primer (5′-CTGGATATCACTGGTGCATCCCAGGGAGAGTGGAGGAAAG-3′), in combination with either of the WT primers, to amplify the same region, and subcloned the products into pBV-Luc.
15. To construct the four tandem repeats of TBE1, TBE2, and TBE2m, we dimerized oligonucleotide cassettes containing two copies of each site and cloned the products into pBV-Luc (for TBE1: 5′-CTAGCGCACCTTTGATTTCTGCACCTTTGATTTCTG-3′ and 5′-CTAGCAGAAATCAAAGGTGCAGAAATCAAAGGTGCG-3′; for TBE2: 5′-CTAGCGGACTCTTGATCAAAGGACTCTTGATCAAAG-3′ and 5′-CTAGCTTTGATCAAGAGTCCTTTGATCAAGAGTCCG-3′; for TBE2m: 5′-CTAGCGGACTCTTGGCCAAAGGACTCTTGGCCAAAG-3′ and 5′-CTAGCTTTGGCCAAGAGTCCTTTGGCCAAGAGTCCG-3′).
16. A glutathione S-transferase (GST)–Tcf-4 fusion protein was constructed by PCR amplification of the sequence encoding the DNA-binding domain (codons 265 to 496) of human Tcf-4 with the following primers: 5′-CGCGGATCCGCTTCCGTGTCCAGGTTCCCTC-3′ and 5′-CGGGAATTCCTAGCCTAGCAGGTTCGGGGAGGG-3′. The PCR product was cloned into pGEX-2TK (Pharmacia). GST–Tcf-4 protein was purified from BL-21 cells, and DNA-binding assays were performed as described [L. Zawel *et al*., *Mol. Cell* **1**, 611 (1998)]. The probes used for TBE1, TBE2, and TBE2m consisted of the oligonucleotides used for construction of multimerized site reporters (*15*). For mutant TBE1m, the following primers were used: 5′-CTAGCGCACCTTTGGCTTCTGCACCTTTGGCTTCTG-3′ and 5′-CTAGCAGAACGCAAAGGTGCAGAACGCAAAGGTGCG-3′. Each binding assay contained 0.5 μg of protein and 0.5 ng of probe end-labeled to 2×10^8 dpm/μg. The specificity of binding was tested by competition with unlabeled WT sites and lack of competition with mutant sites.
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