The histone deacetylase inhibitor butyrate downregulates cyclin B1 gene expression via a p21/WAF-1-dependent mechanism in human colon cancer cells

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The histone deacetylase inhibitor butyrate downregulates cyclin B1 gene expression via a p21/WAF-1-dependent mechanism in human colon cancer cells. Am J Physiol Gastrointest Liver Physiol 289: G696–G703, 2005; doi:10.1152/ajpgi.00575.2004.—Histone deacetylase (HDAC) inhibitors are showing promise as treat-

ments for a variety of human cancers, but their precise mechanism of action has not been elucidated. We examined the effects of the HDAC inhibitor butyrate on colon cancer cells, focusing on its effect on the cell cycle promoter cyclin B1. In HT-29 cells, sodium butyrate-mediated growth inhibition is associated with a marked decrease in cyclin B1 mRNA levels. The decrease in cyclin B1 occurred in a delayed fashion (at 24 h), is completely blocked by concomitant treatment with protein synthesis inhibitors, and appears to be dependent on changes in transcription. Cyclin B1 repression is linked to the differentiation process in colon cancer cells, not merely with growth arrest. The mechanism of cyclin B1 repression by butyrate requires prolonged histone hyperacetylation and is at least partly dependent on p21 expression. In fact, p21/WAF-1 appears to directly repress a minimal cyclin B1 promoter (−90 bp), a process that can be mediated by the amino-terminal portion of the p21 protein. These findings highlight key molecular mechanisms by which HDAC inhibitors mediate their beneficial effects on human cancer cells.

short-chain fatty acids; cell cycle; HT-29; Caco-2

COLON CANCER is the second most common cancer in Western societies, affecting over 135,000 persons each year, and is the third most common cause of cancer deaths in the United States (4). Histone deacetylase (HDAC) inhibitors are emerging as a new and powerful therapeutic strategy in the treatment of a variety of human cancers (6, 48). Butyrate and other short-chain fatty acids (SCFAs) are HDAC inhibitors that are naturally occurring breakdown products of fiber fermentation in the colon and have been found to inhibit the growth of colon cancer cells both in vivo and in vitro (2, 31). We have been interested in understanding the molecular mechanisms that underlie the beneficial effects of HDAC inhibitors and have shown that butyrate and other SCFAs induce growth arrest in colon cancer cells through a process requiring the induction of the G1 cell cycle inhibitor p21/WAF-1 (1). Although most studies have focused on its induction of G1 cell cycle arrest, butyrate also induces a G2 arrest, and the present study was undertaken to examine this aspect of butyrate action.

The major biochemical change that occurs in cells treated with HDAC inhibitors is the global hyperacetylation of histones (38). Histone proteins package DNA into nucleosomes, and core histones can be acetylated on lysine residues of NH2-terminal tails. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferase and HDAC, respectively, with the net level of acetylation controlled by an equilibrium between these enzyme activities (18). Butyrate causes histone hyperacetylation through a noncompetitive and reversible inhibition of HDAC (39). Histone hyperacetylation neutralizes the charge between histone tails and DNA, freeing this region of DNA for access to transcription factors, and is generally associated with activation of specific genes and decrease in cell growth (21, 34). Histone hypoacetylation reverses this process.

Chromatin modification is a key factor in the development of neoplasia because of its importance in the dynamic regulation of transcription. For example, certain oncogenic transcription factors, such as the leukemogenic transcription factors, promote oncogenesis by misregulation of chromatin structure (41). Also, chromatin remodeling is vital to the normal function of tumor suppressors such as Rb (20) and p53, and this function is dysregulated in some cancers. Finally, changes in DNA methylation are prominent characteristics of most cancers, and it is now known that methylated DNA recruits complexes that alter chromatin structure and repress gene transcription (3).

In parallel with the discovery of the importance of chromatin in cancer development was the finding that HDAC inhibitors are potent anticancer agents. These agents, which include sodium butyrate (NaBu) and (R)-trichostatin A (TSA), have displayed antiproliferative and differentiating activity in a wide variety of cancers (30, 32, 44). We (1) have shown that butyrate’s histone hyperacetylation effects are important for the induction of p21 gene expression and growth arrest in colon cancer cells.

Cancer cells are characterized by abnormalities in cell cycle regulation. The mammalian cell cycle progresses through the activity of a variety of protein cyclins (A–H) and their associated cyclin-dependent kinases (cdk) (36). These complexes are inhibited by cyclin/cdk inhibitor proteins, e.g., p21/WAF-1 (40). The balance between the activation and inhibition of cyclin/cdk activities determines whether or not a given cell will proceed through the cell cycle and, as such, may contribute to...
the development and/or progression of neoplasia. Consistent with this idea, it has been shown that B-type cyclins are overexpressed in colon cancers (43). Cyclin B is the major controlling cyclin in the G2 phase of the cell cycle. Two B-type cyclins have been identified in mammalian cells, cyclin B1 (cB1) and B2, and a third, cyclin B3, exists in chickens, frogs, flies, and nematode worms (8, 15, 37). cB1, the first human cyclin identified, is a 62-kDa protein and is the product of a gene encoding a 1.6-kb mRNA (37). B-type cyclins regulate mitosis through activation of a maturation/M phase-promoting factor, cB1-p34cdc2(35). cB1 mRNA levels have been shown to vary throughout the normal cell cycle, with the greatest expression in the G2/M phase and the lowest levels in the G1 phase. During the normal cell cycle, changes in cB1 mRNA levels occur as a result of both transcriptional and posttranscriptional mechanisms (29, 37), and, in addition, the cB1 protein is rapidly degraded at exit from mitosis by a ubiquitin-dependent proteolysis mechanism (16). We (22) have shown that butyrate and other SCFAs decrease expression of cB1 in colon cancer cells. However, the molecular mechanism of this decrease is unknown.

In the present work, we sought to examine the molecular mechanisms underlying the regulation of cB1 by butyrate. We demonstrate in this study that cB1 gene expression in HT-29 colon cancer cells is downregulated by butyrate in a delayed fashion, through a process that involves new protein synthesis and prolonged histone hyperacetylation, and is dependent on the p21 gene.

METHODS

Cell Culture

Human colon carcinoma-derived cell lines HT-29 and Caco-2 were purchased from the American Type Culture Collection (ATCC; Manassas, VA). The cells were maintained in 75-cm² plastic flasks at 37°C and 5% CO2 in DMEM ( Gibco-BRL/Invitrogen; Gaithersburg, MD) supplemented with 10% fetal bovine serum (Sigma-Aldrich Chemical; St. Louis, MO), penicillin-streptomycin (100 U/ml), and 2 mM l-glutamine (standard media). Cells were treated with 5 mM NaBu (Sigma-Aldrich Chemical) or the specific histone deacetylase inhibitor TSA (Sigma-Aldrich) for the times and at the concentrations specified. In some cases, cells were also treated with the protein synthesis inhibitors cycloheximide (10 μg/ml; Sigma-Aldrich Chemical) or anisomycin (5 μg/ml; Sigma-Aldrich Chemical). In some experiments, cells were grown to postconfluence for 7 days. HTC116 p21 wild-type (+/+), heterozygote (+/-), and p21-deleted (-/-) cells [kindly provided by Dr. Bert Vogelstein, Johns Hopkins University, Baltimore, MD (42)] were grown in McCoy’s SA media and treated without or with NaBu.

Northern Blot Analyses

Total RNA was extracted using the guanidinium thiocyanate method (10). Northern blot analyses were then performed by loading 20 μg RNA in each lane of an agarose-formaldehyde gel, separating through electrophoresis, transferring to nitrocellulose membranes, and baking for 2 h at 80°C. cDNA probes were 32P labeled by the random primer method (14), typically to a specific activity of 5 × 10⁸ counts·min⁻¹·μg DNA⁻¹. The cB1 probe is a 1.4-kb NorI/KpnI fragment kindly provided by Dr. Robert Weinberg of the Whitehead Institute for Biomedical Research (37). The p21 probe is a 1.009-kb XhoI/EcoRI fragment derived from human Cip1 cDNA (ATCC). The actin probe is a 1.0-kb PstI fragment derived from mouse β-actin cDNA (11). Conditions for hybridization were as follows: 5× standard saline citrate (SSC)-50% (vol/vol) formamide-1% (wt/vol) SDS at 42°C. Washing conditions were 2× SSC-0.1% SDS at 50°C. Relative changes in mRNA levels were determined by laser densitometry of the autoradiograms and normalized for actin mRNA.

Western Blot Analysis

Total protein was extracted (Promega; Madison, WI), and protein concentration was measured. Protein (100 μg) was separated on SDS-PAGE and transferred to nitrocellulose paper. Blots were probed with primary monoclonal antibodies specific for cB1, p21, and β-actin and horseradish peroxidase-conjugated secondary antibodies (Promega). Relative changes in protein levels were determined by laser densitometry of the autoradiograms and normalized for β-actin protein levels.

Relative Two-Step Real-Time RT-PCR

HT-29 cells at 50–70% confluence were treated with 5 mM NaBu with or without 1 μM actinomycin D (Sigma-Aldrich) for various time periods. At the appropriate time intervals, cells were washed with 1× PBS, total RNA was extracted with TRIzol reagent (Invitrogen), and its concentration was determined by NanoDrop (NanoDrop Technologies; Wilmington, DE). First-strand cDNA was synthesized using 2 μg of total RNA (DNase-treated) in a 20 μl reverse transcriptase reaction mixture using random decamers as per the RETROscript manufacturer’s protocol (Ambion; Austin, TX). Real-Time RT-PCRs were performed in a 25-μl mixture containing a 1/20th volume of cDNA preparation and 1× SYBR green mix (Applied Biosystems; Foster City, CA) with 5 pmol of each primer. A 140-bp amplicon of cB1 was amplified using published primer sequences (27), and a 220-bp of 18S RNA was amplified as an internal control using a forward primer (5′-CCCCCTGAGTCTCTTACGTAAGTG-3′) and a reverse primer (5′-CGCCGTCAGAAATTTCACTCTCCT-3′). Real-time quantifications were performed using a GeneAmp 5700 (Applied Biosystems) sequence detection system, and the fluorescence threshold values were calculated using GeneAmp 5700 software.

DNA Transfer Studies

Plasmid constructs. Various deletional constructs of the cB1 promoter from 1,059 to 64 bp upstream of the transcriptional start site fused to the chloramphenicol acetyltransferase gene were originally obtained from John P. Cogswell (Glaxo Research Institute) (12). The promoter regions were removed using suitable restriction enzymes and subcloned into the pGL3-Basic plasmid (Promega) at the Bgl II site. These plasmids were then used in our experiments.

Transient transfections. Caco-2 cells were seeded in six-well plates at a density that would achieve 70–80% confluence within 24 h. These cells were then transfected at 24 h after being plated. With the use of the Qiagen Superfect transfection protocol (Qiagen; Valencia, CA), HT-29 cells were transiently transfected with pGL3-Basic plasmids containing various deletional constructs of the cB1 promoter from 1,050 to 64 bp upstream of the transcriptional start site with or without the p21 expression plasmid (Pfizer-Pharmacia; New York, NY) or expression plasmids containing the coding regions for the carboxy- or amino-terminals of the p21 protein [gifts of Anindya Dutta, Harvard Medical School, Boston, MA (9)]. The empty pGL3-Basic vector and a similar plasmid containing 2.4 kb of the intestinal alkaline phosphatase (IAP) promoter upstream of its transcriptional start site (22) were used as controls. Luciferase (Luc) assays were performed (Promega), and activity was measured as relative light units and normalized for total protein content of each sample.

Stable transfections. HT-29 cells were seeded in 75-cm² flasks to obtain 70% confluence density in 24 h, when they were transfected using the Qiagen Superfection transfection protocol. Cells were transfected with the Neomycin resistance gene (NEO; 1 μg, Stratagene
Cloning Systems; La Jolla, CA) with or without the p21 expression plasmid (10 μg, Pfizer-Pharmacia) in standard medium. After 48 h, they were transferred to selection G418 media (500 ng G418/ml standard media, Sigma-Aldrich Chemical). Clones were pooled and used for experiments.

Data Analysis

Relative changes in mRNA levels on Northern blots were assessed by laser densitometry of the autoradiograms and normalized for actin mRNA. Statistical analyses were carried out using Student’s unpaired t-test. \( P < 0.05 \) was considered statistically significant.

RESULTS

Butyrate Decreases cB1 Gene Expression

We (23) have previously shown that HT-29 cells induced to differentiate with 5 mM NaBu undergo dramatic growth inhibition with maximal effects at 24 h. Figure 1A shows that concomitant with this growth arrest, expression of cB1 mRNA decreases in a delayed fashion, beginning at 6 h with maximal changes at 24 h (90% decrease, \( P < 0.001 \)). These findings were confirmed by densitometry evaluation (Fig. 1B). This delayed decrease in cB1 expression contrasted to the early induction of p21 mRNA at 2 h, as previously shown (1). Western blot analysis revealed that NaBu-mediated decreases in cB1 protein concentration follow that of mRNA, with maximal decreases at 48 h. Corresponding increases in p21 expression are noted at 24 and 48 h (Fig. 1C). Dose-response experiments revealed that maximum decreases in cB1 mRNA expression were seen at the 5 mM concentration of NaBu (Fig. 1D). This was confirmed by densitometry evaluation (Fig. 1E).

Decreases in cB1 Expression Accompany Cellular Differentiation

Because decreases in cB1 expression by butyrate in HT-29 cells occurred in a delayed fashion, similar to that previously shown for the induction of the differentiation markers IAP and villin (23), we sought to determine whether the dramatic decrease in cB1 was a function of the differentiation process or merely the associated growth arrest. For these studies, we compared mRNA expression of cB1 in postconfluent Caco-2 and HT-29 colon cancer cells because the former cells differentiate under these conditions, whereas the latter cells do not.
Figure 2 shows that, although cB1 mRNA levels decreased dramatically in Caco-2 cells at 7 days postconfluence, there was a minimal change in its expression in postconfluent HT-29 cells, despite the fact that cells were withdrawn from the cell cycle as determined by FACS analysis and [3H]thymidine incorporation (data not shown). These results suggest that the changes in cB1 are specifically linked to the differentiation process and not merely the associated growth arrest.

**cB1 Repression Appears to Occur at the Level of Transcription**

To determine whether the decreases in cB1 expression by butyrate were a result of changes in transcription or mRNA stability, we examined the stability of cB1 mRNA in HT-29 cells in the presence or absence of butyrate. Cells were treated with actinomycin D with or without NaBu, and real-time RT-PCR evaluation of cB1 mRNA expression was determined. As shown in Fig. 3, there was no significant change in the stability of cB1 mRNA in the cells treated with or without NaBu. Thus repression of cB1 by butyrate appears to occur at the transcriptional level.

**cB1 Downregulation Requires New Protein Synthesis**

Figure 4 demonstrates that concomitant treatment with the protein synthesis inhibitors cycloheximide or anisomycin completely blocked NaBu-induced changes in cB1. This requirement for new protein synthesis is similar to that previously demonstrated for the differentiation markers IAP and villin (23) but contrasted with that for the induction of the cell cycle inhibitor p21, which does not require new protein synthesis (1). These results suggest that a new protein(s), e.g., transcriptional repressor, is required for the decrease in cB1 levels caused by butyrate.

**Downregulation of cB1 by NaBu Requires Prolonged Histone Hyperacetylation**

We (45) have previously shown that differentiation in these colon cancer cells requires prolonged histone hyperacetylation, whereas transient histone hyperacetylation is sufficient to induce growth arrest. We therefore compared the effects of transient versus prolonged histone hyperacetylation on cB1 mRNA expression. Acid-urea-triton gel analysis has demonstrated that a single dose of TSA results in transient histone H4 hyperacetylation (6 h), whereas repeated doses of TSA (every 8 h) or a single dose of NaBu caused persistent histone H4 hyperacetylation (45). As shown in Fig. 5, transient histone hyperacetylation did not affect cB1 mRNA expression, whereas prolonged histone hyperacetylation resulted in its downregulation. Therefore, it appears that prolonged but not transient histone hyperacetylation is required for decreases in cB1 mRNA, similar to the mechanism identified for the differentiation process (45).

**p21 Is Important for Repression of cB1 by Butyrate**

We sought to further evaluate the molecular mechanisms underlying cB1 repression by butyrate. Because p21 is induced...
Fig. 6. A: overexpression of p21 protein in stable transfectants. HT-29 control cells were grown in the absence or presence of 5 mM NaBu, and p21 transfectant cells were grown without NaBu. Top, p21 protein expression was examined by Western blot analysis. Bottom, actin control. Representative Western blots are shown (n = 4). B: importance of p21 in expression of cB1. HT-29 control (C), neomycin-expressing control (NEO), and p21-overexpressing cells were grown in standard media for 24 h. p21 (top) and cB1 (middle) mRNA expression were examined by Northern blot analysis. Bottom, actin control. Twenty micrograms of total RNA are present in each lane, with equal loading verified by ethidium bromide staining. Representative Northern blots are shown (n = 4). C: butyrate decreases cB1 mRNA expression in HCT116 p21 (+/+ and p21 (+/-) cells but not in p21 (-/-) cells. HCT116 p21 (+/+), (+/-), and (-/-) cells were grown without (-) or with (+) 2 mM NaBu (higher doses resulted in cell death) for 24 h, and cB1 mRNA levels (top) were evaluated. Bottom, actin control. Twenty micrograms of total RNA are present in each lane, with equal loading verified by ethidium bromide staining. Representative Northern blots are shown (n = 4). D: butyrate decreases cB1 protein expression in HCT116 p21 (+/+ cells but not in p21 (-/-) cells. HCT116 p21 (+/+ and (-/-) cells were grown without (-) or with (+) 2 mM NaBu for 48 h, and cB1 (top) and p21 (middle) protein levels were evaluated. Bottom, actin control. Representative Western blots are shown (n = 4).

before cB1 repression, and because histone hyperacetylation is required for both events, p21 was felt to be a possible mediator of butyrate repression of cB1. To test this hypothesis, we stably overexpressed the p21 gene in HT-29 cells. Western blot analysis revealed that p21 transfectants expressed high levels of p21 protein, similar to those seen in NaBu-treated control HT-29 cells (Fig. 6A). Northern blot analyses also revealed that in the control parent HT-29 cells and NEO cells, there were low levels of p21 expression, whereas p21 stable transfectant cells overexpressed p21 mRNA (Fig. 6B). In control HT-29 and NEO cells, the low basal expression of p21 correlated with high cB1 levels. In contrast, the p21-overexpressing cells (p21) expressed no basal levels of cB1 mRNA whatsoever (Fig. 6B), suggesting that p21 expression may be important in cB1 gene regulation. To further test this hypothesis, we employed HCT 116 p21 wild-type (+/+), heterozygote (+/-), and mutant (-/-) cells. These cells were treated with or without NaBu, and cB1 expression was assessed by Northern blot analyses. Figure 6C shows that, whereas NaBu dramatically decreased cB1 expression in +/+ and +/− cells, there were minimal decreases in −/− cells. Western blot analyses corroborated this data. Figure 6D shows that, whereas NaBu dramatically decreased cB1 while increasing p21 protein expression in +/+ cells, there was no significant decrease in cB1 in −/− cells concomitant with the lack of induction of p21. These results suggest that p21 plays a critical role in butyrate-mediated repression of cB1.

**p21 Represses a Minimal cB1 Promoter**

To define the mechanisms mediating p21 repression of cB1, transient transfections were performed in Caco-2 cells. Various deletional reporter constructs of the human cB1 gene promoter were cotransfected with a p21 expression plasmid. Figure 7A shows that p21 caused at least a 50% decrease in basal expression of all cB1 constructs from 1,050 to 90 bp upstream of the transcriptional start site (P < 0.001). A marked decrease in basal expression was seen in the −64-bp construct, and there

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**Fig. 7.** A: p21 represses a minimal cB1 promoter. cB1 promoter-reporter (Luc) deletional constructs were cotransfected without or with the p21 expression plasmid, and luciferase activity was measured (normalized for total protein). Empty vector (EV) and intestinal alkaline phosphatase-luciferase (IAP) reporter plasmids were used as controls. Representative results are shown (n = 4), normalized for total protein content. Inset: pictorial of various CB1-LUC deletional constructs used in the experiment. B: amino-terminal end of p21 represses cB1 promoter. The 90-bp cB1-Luc construct was cotransfected with expression plasmids containing the coding regions for the carboxy-terminus (C-p21) or amino-terminus (N-p21) of the p21 protein, and luciferase activity was measured (normalized for total protein). Representative results are shown (n = 4), normalized for total protein content.

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was minimal repression by p21. For control purposes, p21 was shown not to affect expression of the empty vector or the IAP-Luc plasmids. These data suggest that p21-mediated repression of cB1 likely occurs through a cis-element located within 90 bp of the transcriptional start site.

As the amino- and carboxy-termini portions of p21 protein are responsible for distinct functions (9), we sought to determine which portion of the protein mediated repression of cB1. The 90-bp cB1-Luc construct, which had been previously shown to undergo significant repression by p21 (see Fig. 7A), was cotransfected with expression plasmids containing the coding region for the p21 amino-terminus (N-p21) or carboxy-terminus (C-p21). Figure 7B shows that, whereas N-p21 significantly repressed cB1 expression (70%, P < 0.001), similar to the full-length p21 as in Fig. 7A, C-p21 had no effect. These results suggest that the amino-terminus of the p21 protein contains the functional domain that mediates cB1 repression.

DISCUSSION

Colon cancer is a particularly prevalent and morbid disease that affects a significant portion of the population in Western countries. Ongoing research studies in colon cancer have been thus targeted toward understanding its pathogenesis and identifying areas of improved treatment. Although several HDAC inhibitors have been identified as possible anticancer agents (30, 32, 44), in regards to colon cancer, butyrate, a SCFA product of fiber fermentation occurring naturally in the colon, is a particularly attractive agent that may have both preventative and therapeutic value. The molecular mechanisms that underlie butyrate’s effects are now beginning to be understood, and it appears that the modulation of chromatin structure through histone hyperacetylation may be the key means by which butyrate mediates its anticancer effects. The present studies suggest that downregulation of cB1 gene expression through the process of histone hyperacetylation may be an important mechanism by which butyrate inhibits the growth of colon cancers.

Although two types of cyclin B are now known to exist in mammals, most studies have focused on the expression and regulation of the first identified cyclin B, cB1, located on human chromosome 5 [region q13-qter (33)]. The precise physiological importance of cB1 is not well known. Interestingly, although cyclin B2-null mice develop normally and are fertile, cB1-null mice die in utero, indicating a critical role for cB1 in normal development (5). cB1 overexpression in several cancers, including that of the colon (43), and its downregulation by the tumor suppressor p53 (25) suggest that cB1 may play an important role in the growth dysregulation accompanying the neoplastic process.

The present studies show that butyrate decreases expression of the cB1 gene in colon cancers cells in vitro. The decrease in cB1 mRNA expression occurs in a delayed fashion (~24 h), is dependent on new protein synthesis, and appears to be a transcriptional effect. This mode of gene regulation is similar to what we have previously shown for the enterocyte differentiation markers IAP and villin and is distinct from that for the p21 cell cycle inhibitor, which is induced in an immediate-early fashion, not dependent on new protein synthesis (1). These decreases in cB1 mRNA by butyrate appear to be specific to its differentiating effects because its expression was essentially unaltered during other conditions of growth arrest, i.e., postconfluent HT-29 cells, but decreased in the differentiating postconfluent Caco-2 cells. The association of cB1 repression and the induction of the differentiated phenotype in colon cancer cells is similar to that described in other cells (24, 26).

It appears that the state of cellular histone acetylation may be a key factor in the prevention or promotion of carcinogenesis. Studies of HDAC inhibitors have proven that they are useful in blocking the growth of various cancer cells, and we and others have shown that induction of the G1 cell cycle inhibitor p21/WAF-1 is critical for this growth arrest (1, 19, 46). The present studies show that histone hyperacetylation plays a key role in the downregulation of cB1 by butyrate. Whereas transient histone hyperacetylation by a single dose of TSA is sufficient to cause an immediate induction of p21 (1), prolonged histone acetylation (repeated doses of TSA) is necessary for the downregulation of cB1, indicating that distinct mechanisms govern the changes in these two cell cycle-related gene products.

It is of interest to speculate how histone hyperacetylation leads to gene repression, as is seen with cB1. Histone deacetylation has been proposed to play a role in cB1 gene repression because the transcription factor Max, which generally heterodimerizes with MAD in a complex involving SIN3 and HDAC, has been shown to repress cB1 expression (13). Katula and others (28) have shown that the cB1 gene is repressed in NIH3T3 fibroblast cells by TSA and that this repression requires different regions of the gene depending on the phase of the cell cycle. Another independent study (7) has shown that overexpression of p21 leads to cB1 repression (7). We extended and advanced these findings in that our data are the first to show that the cell cycle inhibitor p21 represents an important pathway by which butyrate mediates cB1 repression. This repression occurs through a cis-element within 90 bp of the transcriptional start site. Exactly how this repression occurs, i.e., by direct binding of p21 to DNA or through protein-protein interactions, requires further studies. Although p21 is known to contain a zinc finger motif generally seen in transcription factors, it has not thus far been shown to be a transcription factor. Interestingly, however, the amino-terminal portion of the p21 protein, which is required for cB1 repression, binds to cdk complexes. It is possible, therefore, that the cdk complex may be mediating DNA interaction. Others have shown that p53 works through p21 and Rb to downregulate Chk1 (17) and that p21 activates p16INK4 through Sp1 (47). It is interesting to note that p21 was not able to fully repress the cB1 promoter in our studies. This may be explained by a dose-effect phenomenon. In other words, greater amounts of p21 may be required for full repression of cB1. This would be in keeping with the known fact that small quantities of p21 actually promote the cell cycle, whereas greater amounts cause inhibition. Several consensus sequences for various transcription factors have been localized to the 90-bp region upstream of the transcriptional start site, and these include Sp1, heat shock factor (HSF), cap, and NF-Y. One or a combination of the transcription factors may play a role in repression of the ~90-bp region of cB1. Other mechanisms may also be playing a role in cB1 repression, i.e., cB1 repression could occur by both p21-dependent and p21-independent mechanisms. This makes intuitive sense because many stimuli, both internal and external,
converge on and control the G2 checkpoint regulated by cB1. It is important to note that because HT-29 cells are p53 null, butyrate-mediated growth arrest in this cell line is p53 independent.

In conclusion, we found that butyrate, a product of fiber fermentation in the colon, decreases mRNA expression of the cell cycle promoter cB1 in HT-29 human colon cancer cells. These changes are associated with the differentiation of HT-29 cells and require induction of the p21 cell cycle inhibitor, via new protein synthesis in a manner that is dependent on prolonged histone hyperacetylation. p21 is able to repress cB1 gene expression via a minimal 90-bp promoter segment. We have thus identified a novel mechanism by which butyrate may inhibit colon carcinogenesis, a mechanism that may broadly apply to other HDAC inhibitors.

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