

# Sodium butyrate sensitizes TRAIL-mediated apoptosis by induction of transcription from the DR5 gene promoter through Sp1 sites in colon cancer cells

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**Sodium butyrate, a short-chain fatty acid naturally present in the human colon, is able to induce cell cycle arrest, differentiation and apoptosis in various cancer cells. Sodium butyrate is most probably related to the inhibition of deacetylases leading to hyperacetylation of chromatin components such as histones and non-histone proteins and to alterations in gene expression. In this study, we demonstrate for the first time that sodium butyrate selectively up-regulated DR5 but had no effect on the expression of the other TNF- $\alpha$ -related apoptosis-inducing ligand (TRAIL) receptor, DR4. Sodium butyrate-induced expression of DR5 involves the putative Sp1 site within the DR5 promoter region. Using a combination of the electrophoretic mobility shift assay and the luciferase reporter assay, we found that a specific Sp1 site (located at –195 bp relative to the transcription start site) is required for sodium butyrate-mediated activation of the DR5 promoter. When HCT116 cells were incubated with sodium butyrate and TRAIL, enhanced TRAIL-mediated apoptosis was observed. The enhanced apoptosis was measured by fluorescent activated cell sorting analysis, DNA fragmentation, poly (ADP-ribose) polymerase cleavage, down-regulation of XIAP and caspase activity. Taken together, the present studies suggest that sodium butyrate may be an effective sensitizer of TRAIL-induced apoptosis.**

## Introduction

Butyrate is a non-toxic short-chain fatty acid that is produced naturally during the microbial fermentation of dietary fiber in the colon (1). Butyrate plays an important role in homeostasis of the colonic mucosa by inducing pathways of cell maturation, including cell cycle arrest, differentiation and apoptosis (2,3). Sodium butyrate globally suppresses deacetylation of histones, resulting in histone hyperacetylation (4). Many genes are transcriptionally regulated by butyrate, as well as by other histone deacetylase inhibitors (5–7), including trichostatin A. Due to their potent anti-proliferative effects and lack of toxicity, butyrate compounds have received some attention as potential cancer therapeutic agents.

Tumor necrosis factor (TNF)- $\alpha$ -related apoptosis-inducing ligand (TRAIL), also known as Apo2L, is a member of the

TNF family and can induce apoptotic cell death in a variety of cell types including colon cancer cells (8–12). TRAIL binds to four membrane-bound death receptors (DR4, DR5, DcR1, DcR2). Both DR4 and DR5 contain a conserved cytoplasmic region called the ‘death domain’ that is required for TRAIL-induced apoptosis (12–14). However, DcR1 lacks an intracellular domain, and DcR2 contains a truncated death domain. Thus, DcR1 and DcR2 may protect cells from TRAIL-induced apoptosis by acting as decoy receptors (14,15). TRAIL induces apoptosis in a wide variety of tumor cells, but does not cause toxicity to most normal cells. It is supported by the presence of large numbers of decoy receptors on normal cells (14,15). However, recent studies have shown that some cancer cells are resistant to the apoptotic effects of TRAIL (16,17). TRAIL-resistant cancer cells can be sensitized by chemotherapeutic drugs *in vitro*, indicating that combination therapy may be a possibility. Therefore, understanding the molecular mechanisms of TRAIL resistance and ways to sensitize these cells to undergo apoptosis by TRAIL are important issues for effective cancer therapy.

In this study, we examine the potential sensitizing effects of sodium butyrate to TRAIL-mediated apoptosis in human colon cancer cells. Thus, we investigated whether butyrate can be involved in the enhancement of apoptosis mediated by TRAIL. Butyrate treatment results in up-regulation of DR5 expression and renders cells more sensitive to the cytotoxic activities of the TRAIL. We focused on testing Sp1 regulation of butyrate-induced DR5 expression and identified a functional Sp1 binding site that is responsible for regulation of DR5 expression.

## Materials and methods

### Cells and materials

HCT116 and HT29 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The culture medium used throughout these experiments was Dulbecco's modified Eagle's medium, containing 10% fetal calf serum (FCS), 20 mM HEPES buffer and 100  $\mu$ g/ml gentamicin. Sodium butyrate was directly added to cell cultures at the indicated concentrations. Anti-cIAP1, anti-XIAP, anti-cIAP2, anti-Bcl-2, anti-Bcl-xL, anti-PARP, anti-caspase 3, anti-DR5 and anti-Hsc70 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Soluble recombinant TRAIL was purchased from R&D Systems (Minneapolis, MN). Trichostatin A was purchased from Biomol (Plymouth Meeting, PA). Sodium butyrate was obtained from Sigma Chemical.

### Western blotting

Cellular lysates were prepared by suspending  $1 \times 10^6$  cells in 100  $\mu$ l of lysis buffer (137 mM NaCl, 15 mM EGTA, 0.1 mM sodium orthovanadate, 15 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 25 mM MOPS, 100  $\mu$ M phenylmethylsulfonyl fluoride and 20  $\mu$ M leupeptin, adjusted to pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. The proteins were electrophoretically transferred to Immobilon-P membranes (Millipore, Bedford, MA). Detection of specific proteins was carried out with an ECL western blotting kit according to the manufacturer's instructions.

### Cell count and flow cytometry analysis

Cell counts were performed using a hemocytometer. Approximately  $1 \times 10^6$  HCT116 cells were suspended in 100  $\mu$ l of PBS, and 200  $\mu$ l of 95% ethanol was added while vortexing. The cells were incubated at 4°C for 1 h, washed

**Abbreviation:** TRAIL, TNF- $\alpha$ -related apoptosis-inducing ligand.

with PBS, and resuspended in 250  $\mu$ l of 1.12% sodium citrate buffer (pH 8.4) together with 12.5  $\mu$ g of RNase. Incubation was continued at 37°C for 30 min. The cellular DNA was then stained by applying 250  $\mu$ l of propidium iodide (50  $\mu$ g/ml) for 30 min at room temperature. The stained cells were analyzed by fluorescent activated cell sorting (FACS) on a FACScan flow cytometer for relative DNA content based on red fluorescence.

#### DNA fragmentation assay

After treatment with sodium butyrate, TRAIL and a combination of TRAIL and sodium butyrate for 24 h, HCT116 cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1  $\mu$ g/ml of ethidium bromide.

#### Caspase 3 activity assay

To evaluate caspase 3 activity, cell lysates were prepared after their respective treatment with TRAIL or sodium butyrate. Assays were performed in 96-well microtiter plates by incubating 20  $\mu$ g of cell lysates in 100  $\mu$ l of reaction buffer (1% NP-40, 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 10% glycerol) containing the caspase 3 substrate (DEVD-pNA) at 5  $\mu$ M. Lysates were incubated at 37°C for 2 h. Thereafter, the absorbance at 405 nm was measured with a spectrophotometer.

#### Cloning of human DR5 promoters

The pDR5/*SacI* plasmid [containing DR5 promoter sequence (–2500/+3)] and pDR5/–605 [containing DR5 promoter sequence (–605/+3)] were a gift from Dr Sakai T (Kyoto Prefectural University). Point mutations of the Sp1 binding sites to the DR5 promoter were generated by a two-step PCR method using the following primers: mSp1-1 (5'-AGCCGCGAAGTTCCAAGTCAG to 5'-CTGACTTGGAACTTCGCGGCT), mSp1-2 (TTCGCCAAGTTCGGAATGAC to 5'-GTCATTCCGGAAGTTGGCGAA), mSp1-3 (CTTCCAAATCC-AATACCCACTTG to CAAGTGGGTATTTGGATTGGAAG). Triple mutants (mSp1-4, mSp1-5 and mSp1-6) were generated by a two-step PCR method using the same primer, but template DNAs were used as point mutated plasmids. *KpnI* and *NcoI* sites were included in PCR products so that following *KpnI* and *NcoI* digestion of the PCR products they could be subcloned into the pGVB2 *KpnI/NcoI* site. Clones representing each point mutation were sequenced to ensure the accuracy of the PCR amplification procedure.

#### Plasmids, transfections and luciferase gene assays

Sp1 reporter constructs were purchased from Clontech (Palo Alto, CA). In brief, cells were plated onto 6-well plates at a density of  $5 \times 10^5$  cells/well and grown overnight. Cells were co-transfected with 2  $\mu$ g of various plasmid constructs and 1  $\mu$ g of the pCMV- $\beta$ -galactosidase plasmid for 5 h by the Lipofectamine method. After transfection, cells were cultured in 10% FCS medium with vehicle (DMSO) or drugs for 24 h. Luciferase and  $\beta$ -galactosidase activities were assayed according to the manufacturer's protocol (Promega). Luciferase activity was normalized for  $\beta$ -galactosidase activity in cell lysate and expressed as an average of three independent experiments.

#### RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To determine whether the potential sensitizing effects of sodium butyrate to TRAIL-mediated apoptosis were a result of increased levels of mRNA encoding DR4 and DR5, we compared the levels of DR4 and DR5 in HCT116 cells, which were treated with or without various concentrations of sodium butyrate. DR4 and DR5 mRNA expression was determined by RT-PCR. Total cellular RNA was extracted from cells using the TRIzol reagent (Life Technologies). A cDNA was synthesized from 2  $\mu$ g of total RNA using M-MLV reverse transcriptase (Gibco-BRL, Gaithersburg, MD). The cDNA for DR4, DR5 and actin were amplified by PCR with specific primers. The sequences of the sense and antisense primers for DR4 were 5'-AGCTCAG CTGCAACCATCAA-3' and 5'-CACAAACCT GAGCCGATGCAA-3', respectively. The sequences of the sense and anti-sense primer for DR5 were 5'-AAGACC CTTGTGCTCGTTGT-3' and 5'-GACAC-ATTGATGTCACCTCA-3', respectively. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide.

#### Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Preparation of nuclear extracts from control or drug-treated cells was carried out as described previously (18). An oligonucleotide (5'-TTCGCCCGCCCGCAATGAC) corresponding to the Sp1 site was used as the probe. The reaction mixture for EMSA contained 20 mM Tris-HCl, pH 7.6, 1 mM dithiothreitol, 2 mM MgCl<sub>2</sub>, 1 mM EDTA, 10% glycerol, 1% NP-40, 1  $\mu$ g poly(dI-dC) and 5  $\mu$ g nuclear proteins. Unlabeled wild-type oligonucleotide was added into the reaction mixture and incubated for 10 min at room

temperature. <sup>32</sup>P-Labeled probe DNA (300 000 c.p.m.) was added, and the binding reaction was allowed to proceed for another 20 min. For the super-shift assay, 2  $\mu$ g of Sp1 antibody (Santa Cruz Biotechnology) or a control, mouse IgG, were added and incubated for another 2 h. Mixtures were resolved on 8% polyacrylamide gels at 150 V for 4 h. Gels were dried and subjected to autoradiography.

#### Statistical analysis

Data were expressed as means  $\pm$  SE. Statistical significance was evaluated by analysis of variance (ANOVA).  $P < 0.05$  was considered significantly different.

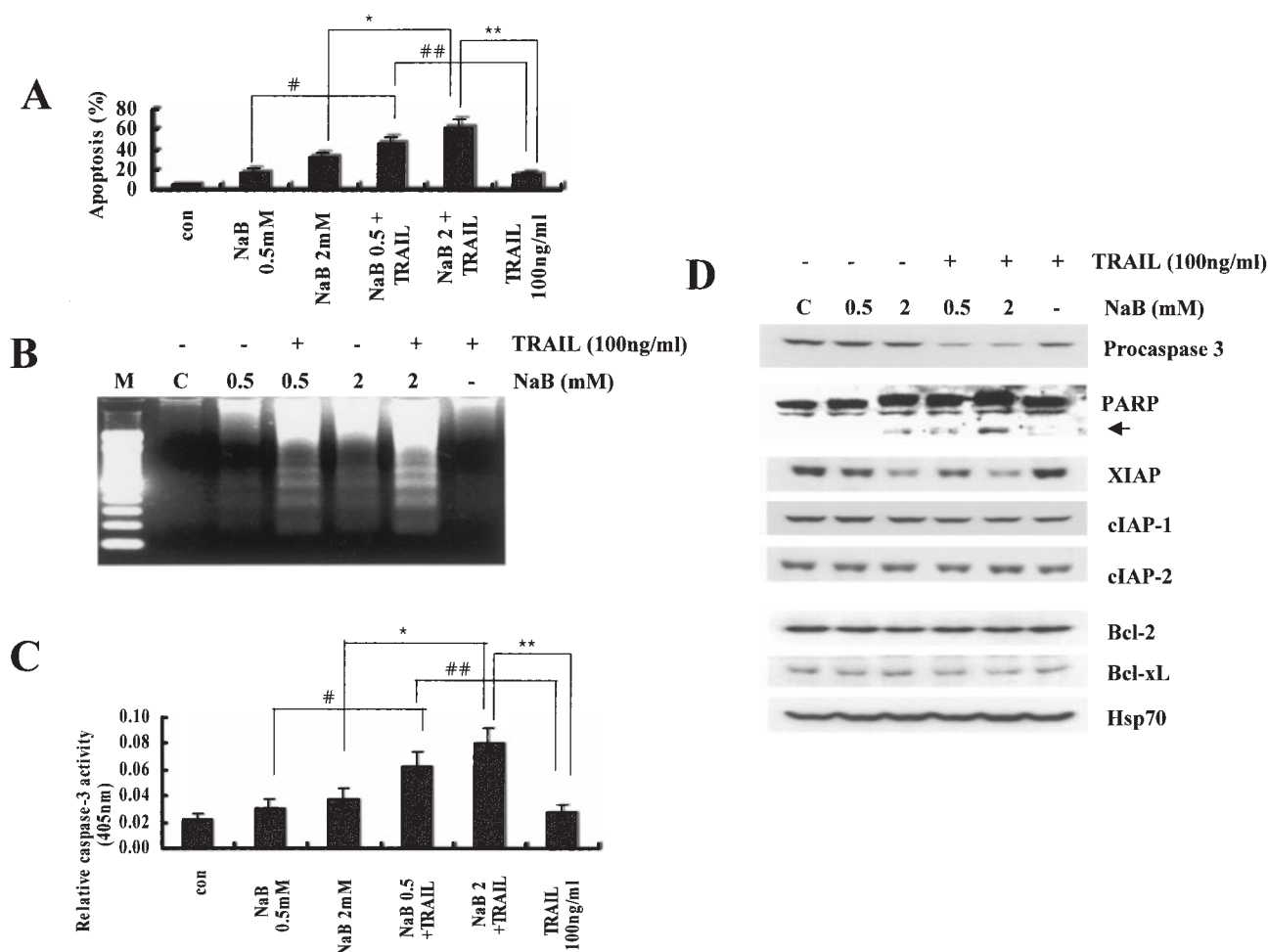
## Results

### Butyrate sensitizes TRAIL-mediated apoptosis

To investigate the effect of butyrate on TRAIL-induced apoptosis, human colon cancer HCT116 cells were treated with sodium butyrate alone (0.5 or 2 mM), TRAIL alone (100 ng/ml), or a combination of butyrate and TRAIL. Three established criteria were subsequently used to assess apoptosis in our system. First, apoptosis was determined in HCT116 cells using flow cytometry analysis demonstrating hypodiploid DNA. As shown in Figure 1A, co-treatment of sodium butyrate and TRAIL in HCT116 cells resulted in a markedly increased accumulation of sub-G<sub>1</sub> phase. Secondly, we analyzed DNA fragmentation, which is another hallmark of apoptosis. Following agarose gel electrophoresis of HCT116 cells treated with sodium butyrate and TRAIL for 24 h, a typical ladder pattern of internucleosomal fragmentation was observed. In contrast, DNA fragmentation in HCT116 cells treated with TRAIL alone or sodium butyrate alone was significantly reduced (Figure 1B). In addition, because cells undergoing apoptosis execute the death program by activating caspases and cleavage of PARP, we analyzed whether co-treatment of sodium butyrate and TRAIL gave rise to the activation of caspase 3, a key executioner of apoptosis. As shown in Figure 1C, co-treatment of butyrate and TRAIL strongly stimulated caspase 3 protease activities. Data of the western blot in Figure 1D showed expression levels of pro-caspase 3, cleavage of PARP, and other apoptosis-related proteins. As shown in Figure 1D, exposure to butyrate and TRAIL led to a reduction of the 32-kDa precursor, accompanied by a concomitant revealed cleavage of PARP. Treatment of HCT116 cells with co-treatment of TRAIL and sodium butyrate for 24 h resulted in a decrease in protein levels of XIAP, but not cIAP1, cIAP2, Bcl-2 and Bcl-xL. Taken together, these results indicate that co-treatment of sodium butyrate and TRAIL sensitizes to induce apoptosis in HCT116 cells.

### Butyrate up-regulates DR5 but not DR4 in colon cancer cells

It is well known that TRAIL can interact with two death receptors (DR4 and DR5), which trigger apoptotic signals. To assess the molecular mechanisms underlying the synergy of butyrate and TRAIL in colon cancer cells, we examined the effect of butyrate on the expression of DR4 and DR5 using RT-PCR. Incubation with butyrate demonstrated induction of the expression of DR5 in HCT116 and HT29 cells, but not DR4. As shown in Figure 2A, butyrate induced the expression of DR5 mRNA in a dose-dependent manner, with a 4.9-fold increase at 4 mM sodium butyrate in HCT116 cells. In HT29 cells, 4 mM sodium butyrate treatments induced a 2.4-fold increase. As the basal level of DR4 mRNA in both cells was much higher than that of the DR5 mRNA, the level of DR4 mRNA in both cell lines was not changed by sodium butyrate. In addition, to examine the butyrate-induced DR5 protein expression in HCT116 cells, cells were exposed to various



**Fig. 1.** Effect of sodium butyrate (NaB) on TRAIL-induced apoptosis in colon HCT116 cells. **(A)** Flow cytometric analysis of apoptotic cells. HCT116 cells were treated for 24 h with the indicated concentrations of sodium butyrate and TRAIL, and their DNA content was measured after propidium iodide staining. The proportion of apoptotic cells is indicated. Data represent the mean  $\pm$  SE for three separate experiments.  $^{\#}P < 0.05$  compared with 0.5 mM NaB;  $^{\#\#}P < 0.05$  compared with 100 ng/ml TRAIL;  $^*P < 0.05$  compared with 2 mM NaB;  $^{**}P < 0.05$  compared with 100 ng/ml TRAIL. **(B)** Fragmentations of genomic DNA in HCT116 cells treated for 24 h with the indicated concentrations of sodium butyrate and TRAIL. Fragmented DNA was extracted and analyzed on 2% agarose gel. **(C)** Cells were treated with the indicated concentrations of sodium butyrate and TRAIL. Equal amounts of cell lysates (40  $\mu$ g) were subjected to electrophoresis and analyzed by western blot for caspase 3, PARP and Hsp70. The proteolytic cleavage of PARP is indicated by the arrow. Enzymatic activities of caspase 3 were determined by incubation of 20  $\mu$ g of total protein with 200  $\mu$ M chromogenic substrate (DEVD-pNA) in a 100  $\mu$ l assay buffer for 2 h at 37°C. The release of chromophore *p*-nitroanilide (pNA) was monitored spectrophotometrically (405 nm). Data represent the mean  $\pm$  SE for six separate experiments.  $^{\#}P < 0.05$  compared with 0.5 mM NaB;  $^{\#\#}P < 0.05$  compared with 100 ng/ml TRAIL;  $^*P < 0.05$  compared with 2 mM NaB;  $^{**}P < 0.05$  compared with 100 ng/ml TRAIL. **(D)** The expression levels of apoptosis related proteins in HCT116 cells by treatment with sodium butyrate and TRAIL. Equal amounts of cell lysates (40  $\mu$ g) were resolved by SDS-PAGE, transferred to nitrocellulose membrane, and probed with specific antibodies, anti-Bcl-2, anti-Bcl-xL, anti-cIAP1, anti-cIAP2 and anti-XIAP, or with anti-Hsp70 antibody to serve as control for the loading of protein level. A representative study is shown; two additional experiments yielded similar results.

concentrations of butyrate and then expression levels of DR5 were determined by western blot analysis. As shown in Figure 2C, butyrate induced the expression of DR5 protein in a dose-dependent manner.

#### *Sodium butyrate activates transcription from the DR5 gene promoter*

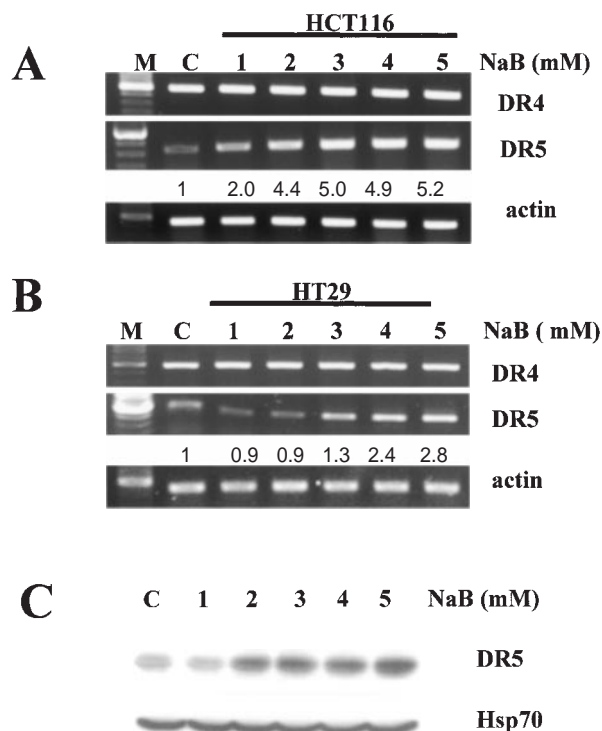
To understand further the molecular mechanisms underlying the effect of butyrate on DR5 gene expression, we employed the luciferase gene expression system. The 2.5 and 0.6 kb of the promoter region of human DR5 gene was cloned into the pGL2-basic luciferase reporter vector, and the resultant was named as pDR5/*SacI* and pDR5/-605, respectively (19). HCT116 cells were transfected with these promoters and the luciferase activity was assayed after 24 h of the various concentrations of sodium butyrate treatment. Sodium butyrate

significantly increased pDR5/*SacI* promoter activity in a dose-dependent manner (Figure 3). pDR5/-605 has 2-fold higher activity than that of pDR5/*SacI* in absence of butyrate.

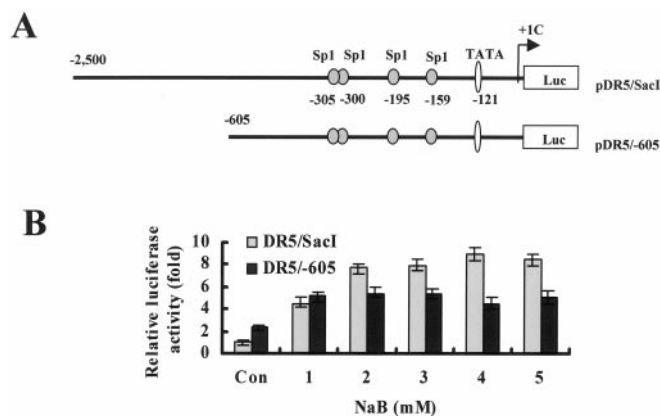
#### *The Sp1 site is required for sodium butyrate-induced transactivation of DR5 in HCT116 cells*

Yoshida *et al.* and our studies identified that pDR5/-605 retained near full luciferase activity (19). The region spanning -605 to +3 contains four Sp1 sites and a TATA-like box site as typical transcription factor binding sites (Figure 3). In order to further confirm the functional role of Sp1 on DR5 promoter activities, we then tested the activities of these transcription factors regulated by sodium butyrate using Sp1 reporter vectors, which have three Sp1 binding sites. Sp1 activities were significantly increased by treatment with sodium butyrate in a dose-dependent manner (Figure 4A). To further confirm





**Fig. 2.** The expression levels of the DR5 mRNA and DR5 protein by treatment with sodium butyrate in colon cancer cells. HCT116 (A) and HT29 (B) cells were treated with the indicated concentrations of sodium butyrate. Total RNA was isolated and RT-PCR analysis was performed. The values below the figure represent change in mRNA expression of the bands normalized to actin. A representative study is shown; two additional experiments yielded similar results. (C) HCT116 cells were treated with the indicated concentrations of sodium butyrate. Equal amounts of cell lysates (40  $\mu$ g) were subjected to electrophoresis and analyzed by western blot for DR5 or anti-Hsp70 antibody to serve as control for the loading of protein level.



**Fig. 3.** Effects of sodium butyrate on the DR5 promoter activities. (A) Schematic structure of DR5 promoter constructs used for testing luciferase activity. (B) pDR5/SacI or pDR5/-605 promoter plasmid was transfected, and treated with varying concentrations of sodium butyrate. The cells were lysed and luciferase activity measured. Data represent the mean  $\pm$  SD of at least three independent experiments.

the functional role of this sodium butyrate-mediated DR5 promoter activation, HCT116 cells were transfected with the Sp1 cDNA, and these cells were treated with sodium butyrate. As shown in Figure 4B, ectopic expression of Sp1 significantly

increased the promoter activity in pDR5/SacI and pDR5/-605. To decipher which Sp1 site plays a critical role in butyrate-mediated activation of DR5/-605 promoter, several Sp1 mutants of the promoter were made and tested in the transfection assay (Figure 4C). As shown in Figure 4C, the introduction of mSp1-1 (mutated at -159 Sp1 site) and mSp1-2 (mutated at -195 Sp1 site) construct decreased butyrate-mediated DR5 promoter activity compared with the wild-type (pDR5/-605) construct. The DR5 promoter activity derived from the double (mSp1-4) and triple mutant constructs (mSp1-5) were significantly decreased by butyrate treatment. However, a triple mutant construct (mSp1-6; mutated at -159, -300 and -305 Sp1 sites) and a double mutant mSp1-3 (mutated at -300 and -305 Sp-1 sites) had only a slight effect on DR5 promoter activity. These results suggest that the -195 Sp1 site is important for butyrate-mediated DR5 promoter up-regulation.

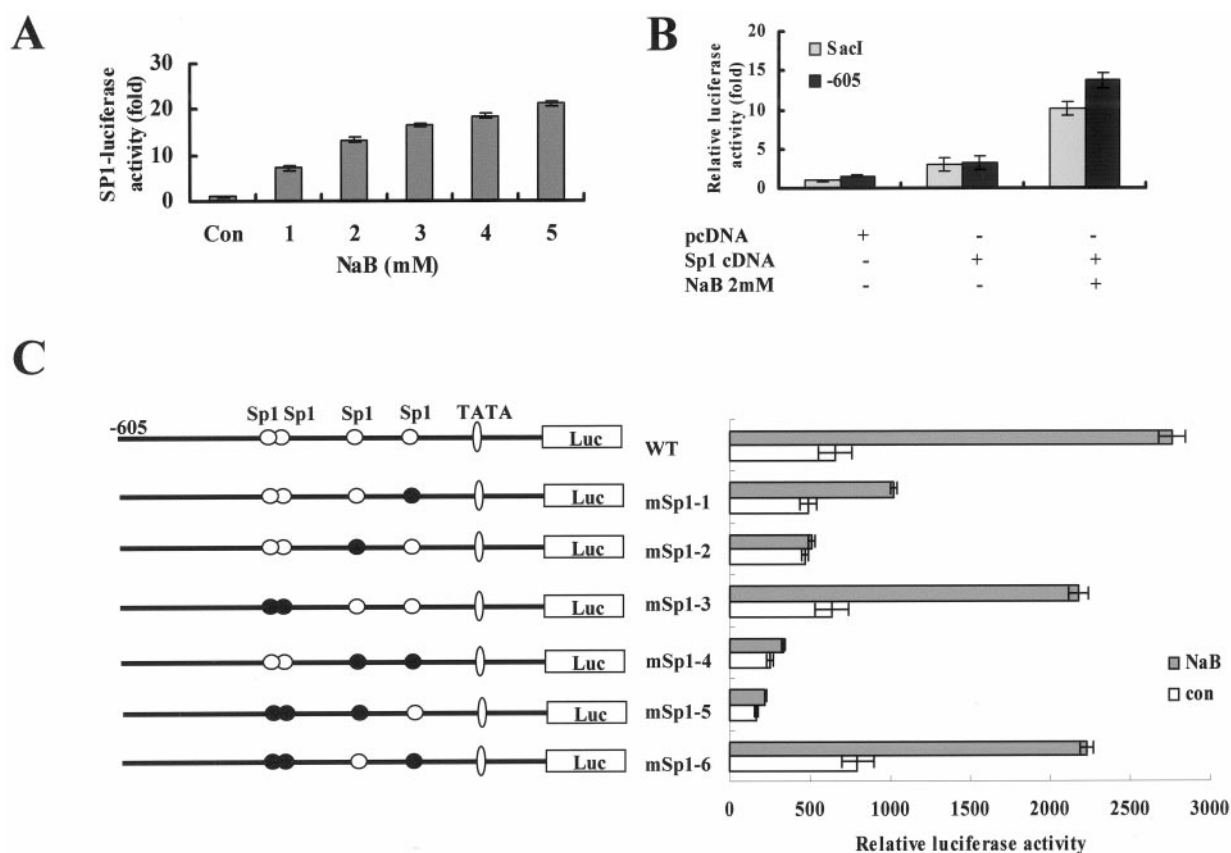
To confirm the involvement of Sp1 in the sodium butyrate response, electrophoretic mobility shift assays were performed with a labeled synthetic double-stranded DNA probe containing the putative Sp1-binding sequence present at -201/-182 (Figure 5A). This sequence was chosen as the probe because mutations within this sequence resulted in the greatest reduction in transcription. A consensus Sp1 oligonucleotide significantly reduced the binding of nuclear proteins to the labeled DNA, whereas the mutant sequence had no effect on the binding. The identity of the Sp1 protein in this complex was further confirmed by a super-shift assay using an antibody specific to Sp1 (Figure 5B). The intensity of the Sp1 signal was greater in sodium butyrate-treated cells than in control cells, suggesting an increased affinity probably of Sp1 nuclear proteins for the labeled DNA in the sodium butyrate-treated cells (Figure 5A).

#### Effect of trichostatin A on DR5 expression

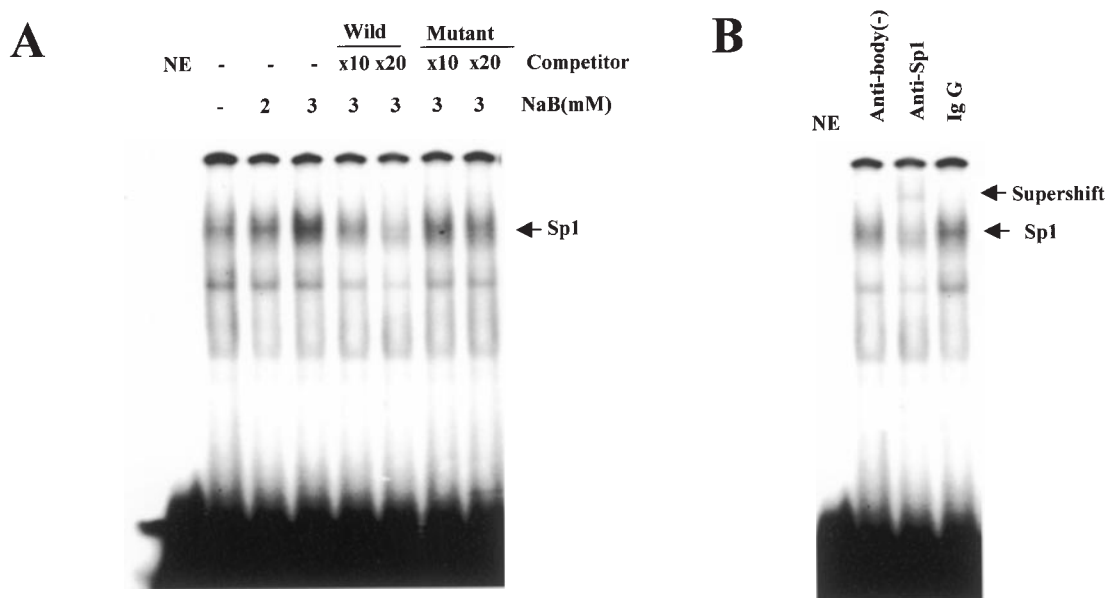
Sodium butyrate inhibits histone deacetylation (4,5). This effect is the basis for the long-standing concept that the action of sodium butyrate on gene transcription is related to alteration on chromatin structure resulting from butyrate-induced hyperacetylation of histones (4,5). Therefore, we determined the influence of histone hyperacetylation on the overall activation of transcription from the DR5 gene promoter, by assessing the effect of trichostatin A, which is a potent histone deacetylase inhibitor (20). As shown in Figure 6A, trichostatin A induced the expression of DR5 mRNA in a dose-dependent manner, with a 3.7-fold increase at 500 nM trichostatin A in HCT116 cells, a pattern almost similar to that seen with sodium butyrate (see Figure 2A). Figure 6B also shows that trichostatin A activated transcription from the DR5/SacI promoter in a dose-dependent manner, when used at up to 500 nM. This pattern was essentially similar to that noted for either trichostatin A (Figure 6B) or sodium butyrate (Figure 3), suggesting some commonality in the mode of action of these compounds in inducing transcription from the DR5 gene promoter.

#### Discussion

We have demonstrated that treatment of colon cancer cells with histone deacetylase inhibitor sodium butyrate in combination with TRAIL synergistically induced apoptosis. The mechanism of this synergy involves the sodium butyrate-induced expression of DR5 but not DR4 that is mediated in part by activation of Sp1 transcriptional factor.



**Fig. 4.** Mutational analysis of transcriptional regulatory elements in the DR5 promoter region. (A) Effects of sodium butyrate on the activities of Sp1. To elucidate the effects of sodium butyrate on Sp1 activities, a reporter vector (pSp1-luc) that has three Sp1-binding sites was transfected. The cells were treated with or without varying concentrations of sodium butyrate, and lysed and luciferase activity measured. (B) HCT116 cells were co-transfected with Sp1 cDNA and DR5 promoter plasmids. The cells treated with sodium butyrate, and lysed and luciferase activity measured. (C) Nucleotides of the Sp1 consensus site in the promoter region were substituted as described in Material and methods. HCT116 cells were transfected transiently with clones in left panel. The extracts from cells treated with or without sodium butyrate were assayed for luciferase activity. Data represent the mean  $\pm$  SD of at least three independent experiments.

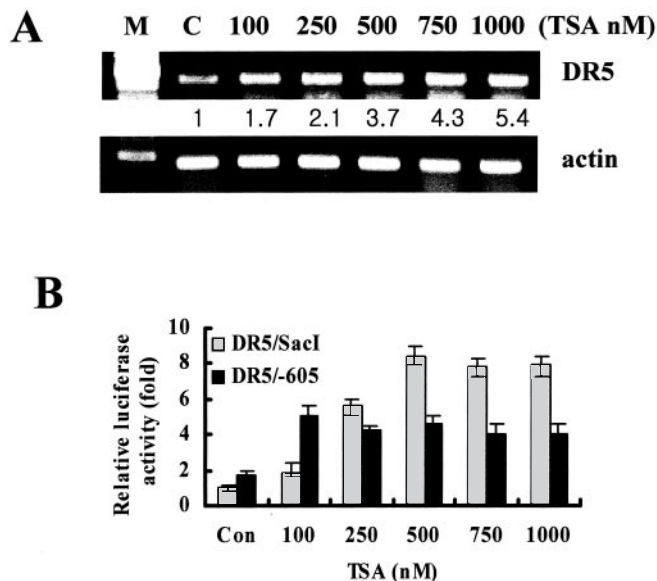


**Fig. 5.** Effect of sodium butyrate on Sp1 DNA binding activity. (A) HCT116 cells were treated with or without various concentrations of sodium butyrate for 6 h. Nuclear extracts were prepared and analyzed in an electrophoretic mobility assay with a radiolabeled Sp1 probe. Equal amounts (5  $\mu$ g) of nuclear proteins were loaded in each lane. Binding specificity was determined using the unlabeled wild-type probe or mutant-type containing the Sp1 binding sequence (the putative Sp1-binding sequence present at -201/-182) to compete with the labeled oligonucleotide. (B) To identify whether the bound proteins contain Sp1, super-shift analysis was performed using an antibody specific to Sp1. Super-shift with a mouse IgG was used as a negative control.

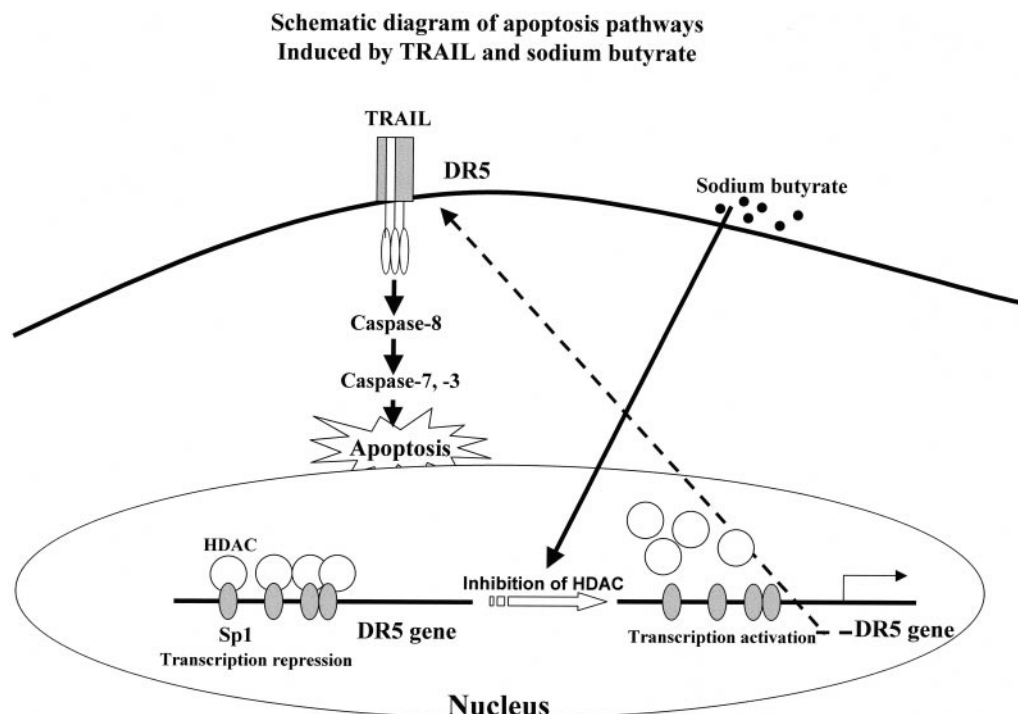
TRAIL is a recently identified member of the TNF family and is capable of inducing apoptosis in various tumor cells (9,12). TRAIL induces apoptosis by interacting with two death-inducing receptors DR4 and DR5. The death receptors DR4 and/or DR5 have been demonstrated to play a crucial role

in synergistic cytotoxicity with TRAIL and chemotherapeutic agents. Several reports have shown that chemotherapeutic agents and ionizing radiation can sensitize TRAIL-induced cytotoxicity by decreasing intracellular levels of FLICE-inhibitory protein (FLIP) or increasing DR5 gene expression. FLIP structurally resembles caspase-8 but lacks proteolytic activity, thus functioning as a dominant-negative inhibitor of caspase-8 (21–23). Recently, two groups have reported that butyrate decreases FLIP expression and enhances TRAIL-induced apoptosis in colon cancer (24,25).

In our present study, we demonstrated that sodium butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis. Caspases are critical protease mediators of apoptosis triggered by various apoptotic stimuli. Interaction of TRAIL with death receptors DR4 and/or DR5 activates the caspase cascade, resulting in apoptosis (26). We found that the combination of butyrate and TRAIL caused increase of caspase 3 activity, down-regulation of XIAP and PARP cleavage. Butyrate is a well-recognized global histone deacetylase inhibitor, although it is also capable of histone methylation (27). We now show that in colon cancer cell lines, HT29 and HCT116, DR5 expression is similarly up-regulated in a dose-dependent manner by both butyrate and trichostatin A. The butyrate-activated transcription of many genes involves Sp1 sites (28–30). For example, sodium butyrate activation of transcription from the galectin-1 gene, mitochondrial HMG-CoA synthase gene and p21<sup>Waf1</sup> gene promoter involves an Sp1 site proximal to the transcription start site (28–30). Sodium butyrate induced expression of DR5 involves the putative Sp1 site within the 5'-untranslated region (19). Using a combination of the EMSA and the luciferase reporter assay for analysis of the importance of each Sp1 binding site, we demonstrated that the putative Sp1 binding site located at –201/–182 was functionally active. Although we showed that mutating the –195 Sp1 site significantly decreases sodium butyrate



**Fig. 6.** Effect of trichostatin A on DR5 expression. (A) HCT116 cells were treated with various concentrations of trichostatin A. Total RNA was isolated and RT-PCR analysis was performed. The values below the figure represent change in mRNA expression of the bands normalized to actin. A representative study is shown; two additional experiments yielded similar results. (B) pDR5/SacI or pDR5/–605 promoter plasmid was transfected, and treated with varying concentrations of trichostatin A. The cells were lysed and luciferase activity measured. Data represent the mean  $\pm$  SD of at least three independent experiments.



**Fig. 7.** Tentative model for the mechanism of TRAIL and sodium butyrate-induced apoptotic pathways.

responsiveness, we cannot rule out the possibility that another Sp1 site may be involved in mediating sodium butyrate-dependent activation of the DR5 under certain circumstances. However, sodium butyrate strongly increased the binding of this Sp1 site by nuclear extracts from butyrate-treated cells and the luciferase activity of the reporter construct containing this site. Therefore, we conclude that Sp1 is involved in the regulation of DR5 expression via the Sp1 binding site located at -201/-182.

Up-regulation of DR4 and DR5 enhanced the responsiveness of cells to TRAIL (9). TRAIL itself up-regulates DR5 mediated by NF- $\kappa$ B activation in epithelial-derived cells, while DR4 expression remains unchanged (31). DR5 has been shown to be induced by DNA damaging agents and radiation in a p53-dependent manner (32,33). In contrast, DR5 is regulated by chemotherapeutic drugs independent of p53 status (34). Since detailed analysis of DR5 expression in both cancer cells and human development is still unknown, we believe cloning of the promoter region provides a useful tool to study the expression of the DR5 gene during cancer progression.

Taken together, these results suggest, in part, that sodium butyrate sensitizes human colon cancer cells to TRAIL-mediated apoptosis by down-regulation of XIAP and up-regulation of DR5 expression that is mediated by activation of Sp1 transcriptional factor. Based on these results, we propose a model that is one of the mechanisms in which butyrate sensitizes TRAIL-mediated apoptosis (Figure 7). In this model, Sp1 may serve as a critical transcriptional factor in sodium butyrate-induced DR5 expression. Inhibition of histone deacetylase activity by sodium butyrate or trichostatin A disrupted the association of histone deacetylase with Sp1, which in turn leads to the decondensation of local chromatin and activates the transcription of DR5. Sodium butyrate treatment results in the up-regulation of DR5 and renders cells more sensitive to the cytotoxic activities of TRAIL. Studies to understand the regulation of transcription of the DR5 gene may be useful for screening the regulators of DR5 gene expression, and for analyzing these regulatory mechanisms in the hope that this will lead to novel cancer therapy.

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