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Activation of CMV promoter-controlled glycosyltransferase and β-galactosidase glycogenes by butyrate, tricostatin A, and 5-Aza-2′-deoxycytidine

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Cytomegalovirus (CMV) immediate early promoter is a powerful promoter frequently used for driving the expression of transgenes in mammalian cells. However, this promoter gradually becomes silenced in stably transfected cells. We employed Chinese Hamster Ovary (CHO) and human pancreatic cancer (Panc 1) cells stably tansfected with three glycogenes driven by a CMV promoter to study the activation of silenced glycogenes. We found that butyrate, tricostatin A (TSA), and 5-aza-2′-deoxycytidine (5-Aza-dC) can activate these CMV-driven glycogenes. The increase in mRNA and protein of a glycogene occurred 8–10 h after butyrate treatment, suggesting an indirect effect of butyrate in the activation of the transgene. The enhanced expression of the trangenes by butyrate and TSA, known inhibitors of histone deacetylase, was independent of the transgene or cell type. However, the transgene can be activated by these two agents in only a fraction of the cells derived from a single clone, suggesting that inactivation of histone deacetylase can only partially explain silencing of the transgenes. Combination treatment of one or both agents with 5-Aza-dC, a known inhibitor of DNA methylase, resulted in a synergistic activation of the transgene, suggesting a cross-talk between histone acetylation and DNA demethylation. Understanding the mechanisms of the inactivation and reactivation of CMV promoter-controlled transgenes should help develop an effective strategy to fully activate the CMV promoter-controlled therapeutic genes silenced by the host cells.
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Introduction

Viral promoters, such as the cytomegalovirus (CMV) immediate early promoter and the simian virus 40 promoter, have been widely used for promoting transgene expression in mammalian cells *in vitro* and *in vivo* because of their potent promoter activity. However, many transgenes under the control of viral promoters have been found to be silenced [1,2]. For example, the CMV promoter is often gradually suppressed over a period of months in culture [3]. The mechanism of silencing the CMV promoter is not clear although histone hypoacetylation and DNA methylation have been suggested [4].

Butyrate and Trichostatin A (TSA), which are known inhibitors of histone deacetylase, have been shown to reactivate the suppressed viral promoters [5–7]. Reactivation of CMV promoter after TSA treatment was preceded by acetylation of specific lysine residues in histone 4 at the transgene locus [7]. It is also known that treatment of 5-aza-2′-deoxycytidine (5-Aza-dC), an inhibitor of DNA methylase, activates genes silenced in cancer [8]. Butyrate and TSA can induce genome wide demethylation, also [8,9]. Furthermore, TSA can induce demethylation of transiently transfected genes that were ectopically methylated [10]. Another inhibitor of histone acetyltransferase, Set/TAF-1β, has been shown to inhibit active demethylation of DNA [11]. Taken together, these findings suggest that inactivation of foreign genes can occur by inactivation of histone acetylation and DNA methylation, which may be reversed by treatment with butyrate, TSA, and 5-Aza-dC.

In this paper, we employed butyrate, TSA, and 5-Aza-dC to study activation of silenced genes under the control of the CMV promoter in two different cell lines stably transfected with three CMV promoter-driven glycogenes, including β-galactosidase and two glycosyltransferases. We found that all three agents

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can reactivate the silenced genes. It took about 8–10 h of butyrate treatment before the enhanced transgene expression was detected, suggesting an indirect effect of butyrate. A combination treatment with butyrate and TSA activated the transgene expression slightly more than either one, suggesting sharing of major mode(s) of action for these two agents and possession of some property unique to each agent. Finally, synergistic effect was observed when combining either agent or both with 5-Aza-dC, suggesting a cross talk between histone acetylation and DNA demethylation in gene activation.

Material and methods

Construction of expression vector and stable cell line generation

Bovine (b) C2GnT-1(or L) [12] and –2(or M) [13] were cloned into mammalian expression vectors, pcDNA3/*Zeocin* and pcDNA6/Myc-His/*bsd* (Invitrogen, Carlsbad, CA) to generate pc3-bC2GnT-L and pc6-bC2GnT-M, respectively. pcDNA 3/*Zeocin* and pcDNA6/Myc-His/*bsd* are mammalian expression vectors with the CMV promoter and bovine growth hormone polyadenylation signal. pc3-bC2GnT-L was transfected into CHO and Panc1/MUC1 cells [14], and pc6-bC2GnT-M was stably transfected into Panc1/MUC1 cell using a transferrin-assisted lipofection protocol as previously described [15]. Stable clones were selected based on resistance to the antibiotic zeocin (300 μg/ml) or blasticidin (10 μg/ml). CHO-LacZ cells were generated by transfection of pcDNA6/LacZ/*bsd* and subsequent selection of blasticidin-resistant clones.

Cell culture

CHO cells were grown in F12 Ham/10% FBS and Panc1/MUC1 cells in MEM/5% FBS. Panc1/MUC1 cell is a human pancreatic carcinoma cell line stably expressing MUC1 tandem repeat as previously described [16].

β-1,6-*N*-acetylglucosaminyltransferase assay

C2GnT activity was assayed using total cell lysates. Confluent cells from a T25 flask were scrapped off the flask in 250 μl of 0.25 M sucrose, and disrupted by forcefully passing the cells successively through 20- and 25-gauge needles. A 50 μl reaction mixture was prepared by adding 29 μl of the cell lysate to the mixture containing the followings: 50 mM MOPS (pH 7.5), 5 mM $MnCl_2$, 2% Tween-20, 1 mM ATP, 1 mg/ml BSA, 2 mM UDP-[^{3}H]GlcNAc ($\sim$1500 dpm/nmol, American Radiolabeled Chemicals, St. Louis, MO), and 2 mM Galβ1-3GalNAcα-*O*-Benzyl (Toronto Research Chemicals Inc. Downsview, Ontario, Canada). The reaction mixtures were incubated at 37°C for 2 h and terminated with 0.3 ml of 10 mM $ZnCl_2$. The products were isolated by solid phase extraction on C18 cartridges with 4 $\times$ 0.5 ml of methanol as previously described [17]. The isolated products were dried by Automatic Environmental SpeedVac System (Savant, Holbrook, NY) and re-suspended in 500 μl of H_2O. Radioactivity of the incorporated sugars was measured by Liquid Scintillation Counter (Packard, Meriden, CT). Protein concentration was measured using Coomassive Plus Protein Assay Reagent (Pierce, Rockford, IL) with BSA as the standard. Enzyme activity was calculated by subtracting the endogenous activity measured without exogenous acceptor from total activity and was expressed as nmol of sugar donor transferred/h/mg protein.

Western blot analysis

CHO/bC2GnT-L cells in T25 flask were grown until 80–90% confluence and treated with 2 mM sodium butyrate for up to 24 h. Cell lysates were resolved in SDS-polyacrylamide gel (4% stacking and 6% separating gels) electrophoresis. Proteins were electroblotted onto PVD membrane (Immobilon-P, 0.45 μ, Millipore, Bedford, MA) overnight at 100 mA, then blocked with 5% nonfat milk in TBS (0.9% NaCl, 10 mM Tris, pH7.5) at room temperature for 1 h. The membranes were then incubated for 1 h at room temperature with bC2GnT-L antibody (1:500 dilution) [12] in 5% nonfat milk. The membranes were washed with 5% nonfat milk in TBS for 15 min and then 5 min twice. The membranes were incubated with peroxidase-conjugated goat anti-mouse IgG secondary antibodies (1:2000 dilution) in TBS containing 5% nonfat milk at room temperature for 1 h. The membranes were washed as described above and rinsed with TBS. ECL reagents (Pierce, Rockford, IL) were applied per the manufacturer' instruction and the blots were exposed to ECL-sensitive film (Amersham Pharmacia Biotech, Uppsala, Sweden).

Northern blot analysis

Two T25 flasks of 80% confluent CHO-bC2GnT-L cells were treated with 2 mM sodium butyrate, 1 μM TSA, or both for various periods as indicated in each experiment before harvest. Total RNAs were isolated from one of the two T25 flasks using Tri Reagent (MRC Inc., Cincinnati, OH). Twenty-five μg of the total RNA were fractionated by glyoxyal-1% agarose gel electrophoresis and transferred to a nitrocellulose membrane and UV cross-linked. The membrane is prehybridized in 50% formamide, 5X saline sodium citrate (SSC), 5X Denhardt's solution, 5 mM EDTA, 0.1% SDS, and 100 μg/ml denatured salmon sperm DNA at 42°C for 4 h. The hybridization probe was labeled with [α-^{32}P]dCTP (ICN, Costa Mesa, CA) using Prime-It II Random Primer DNA Labeling Kit (Stratagene, Cedar Creek, TX) and hybridized in the prehybridization solution supplemented with 10% dextran sulfate at 42°C for 14 h. The membrane was washed once with 0.1% SDS in 2X SSC at room temperature for 15 min and twice with 0.1% SDS in 0.2X SSC at 58°C for 15 min and exposed to X-OMAT AR film (Kodak, Rochester, NY) with intensifying screen at –80°C for 48–72 h. The membrane was subsequently rehybridized with glyceraldehyde 3-phosphate dehydrogensae (GAPDH) probe as an internal control for equivalent RNA loading.

β-Gal assay and X-gal staining

The expression of β-Gal gene was assessed by measuring β-Gal activity and % blue cells following 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. β-Gal activity was measured using Mammalian β-Gal Assay Reagent Kit (Pierce). Cells grown to 80–90% confluence in 48-well plate were treated with various combinations of NaBu, TSA, and 5-Aza-dC (1 μg/ml) for 24 h before lysed with 150 μl of M-PER Mammalian Protein Extraction Reagent for 10 min with gentle shaking. Ten and 20 μl of the lysates were used for β-Gal reaction with 100 μl of All-in-One™ β-Gal Assay Reagent and the volume of reaction mixture was adjusted to 200 μl with H_2O. The reaction mix was incubate at 37°C for 30 min, and then stopped by adding 100 μl of stop solution (0.5 M $NaHCO_3$). β-Gal activity in the reaction mixture was measured by reading at 404 nm with UVmax microplate reader (Molecular Devices, Sunnyvale, CA), which was converted to the amount of β-galactosidase using E. coli β-galactosidase as the standard. The enzyme activity was expressed as ng β-galactosidase/μg protein.

For examination of the effects of NaBu and TSA on the activation of stably transfected β-galactosidase gene in colonies derived from a single clone, the cells derived from a single clone were extensively diluted before plated onto a 100 mm dish. After discrete colonies were formed, the cells were treated with NaBu or TSA for 24 h before being fixed in ice-cold 2% paraformaldehyde-0.2% glutaraldehyde for 10 min. The cells were washed twice with Hepes-buffered saline and then exposed to 1.0 mg/ml X-Gal at 37°C overnight as previously described [15,18]. Three independent fields were counted in each treatment plate and the data were expressed as the mean ± SE. The pictures of blue cells were taken under a phase-contrast microscope equipped with a digital camera.

Statistical analysis

Statistical analysis was performed using a two-tail *t*-test method (GrapPad). $P < 0.05$ was considered a significant difference of means between groups.

Results and discussion

Sodium butyrate enhances the expression of pCMV-bC2GnT-L and -M cDNAs stably transfected in mammalian cells

The mammalian cells that contain bC2GnT-L/-M glycogenes under the control of the CMV promoter used in this study included CHO-bC2GnT-L, Panc1-bC2GnT-L, and Panc1-bC2GnT-M cells. The basal C2GnT activities in bC2GnT-L stable (both CHO and Panc 1 cells), Panc 1-bC2GnT-M stable, and Panc 1 cells used in this study were 30–45, 185, and 2 nmole/h/mg protein, respectively (Figure 1). C2GnT activities in two additional Panc 1-bC2GnT-M clones, which expressed lower enzyme activities than the one described above, were also

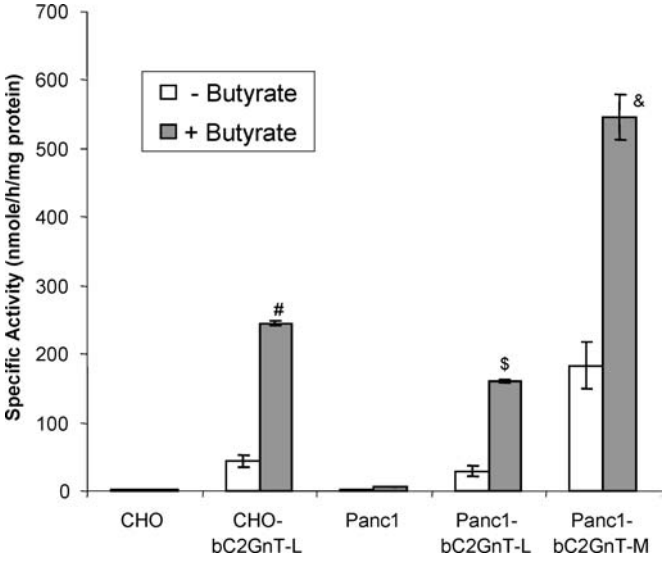

Figure 1. Effects of butyrate treatment on enhancing C2GnT activity in CHO, CHO-bC2GnT-L, and Panc1-bC2GnT-M cells. These cells were treated with 2 mM sodium butyrate for 24 h before analysis of C2GnT activity ($n = 3$). C2GnT activity in Panc1 cells was 2 and 6 nmole/h/mg protein before and after butyrate treatment. CHO cells did not have any detectable C2GnT activity under the assay conditions used even after butyrate treatment. #, $p = 0.0001$ for CHO-bC2GnT-L cells treated with vs. without butyrate; $, $p = 0.005$ for Panc1-bC2GnT-L cells treated with vs. without butyrate; and &, $p = 0.007$ for Panc1-bC2GnT-M cells treated with vs. without butyrate.

stimulated 3 fold by butyrate (data not shown). Thus, butyrate treatment for 24 h increased C2GnT activity three to five fold in all stably transfected cells. The C2GnT activity in parental CHO cells was not detectable under the assay conditions described even after butyrate treatment. However, activation of endogenous C2GnT activity in CHO cells by butyrate was previously reported using a very sensitive C2GnT assay procedure [19].

To determine the kinetics of the butyrate effect on transgene expression, bC2GnT-L transcript, protein, and enzyme activity in pCMV-bC2GnT-L cDNA stably transfected CHO cells treated with sodium butyrate were measured for up to 24 h (Figure 2). The bC2GnT-L messages began to increase after butyrate treatment for 8 to 10 h and continued to increase until reached more than 20 fold of the control value after 24 h while GAPDH message was not changed (Figure 2). The relative amounts of bC2GnT-L protein estimated by western blotting closely followed the level of bC2GnT-L messages. The C2GnT enzyme activity started to increase after butyrate treatment for 8–10 h and approached the plateau after 24 h. These results strongly suggest that the enhancement of bC2GnT-L expression by sodium butyrate is due to increased transcriptional activity of the CMV promoter. The relatively long period of time, *i.e.* 8–10 h, after butyrate treatment before the enhancement of C2GnT expression is detected suggests an indirect nature of

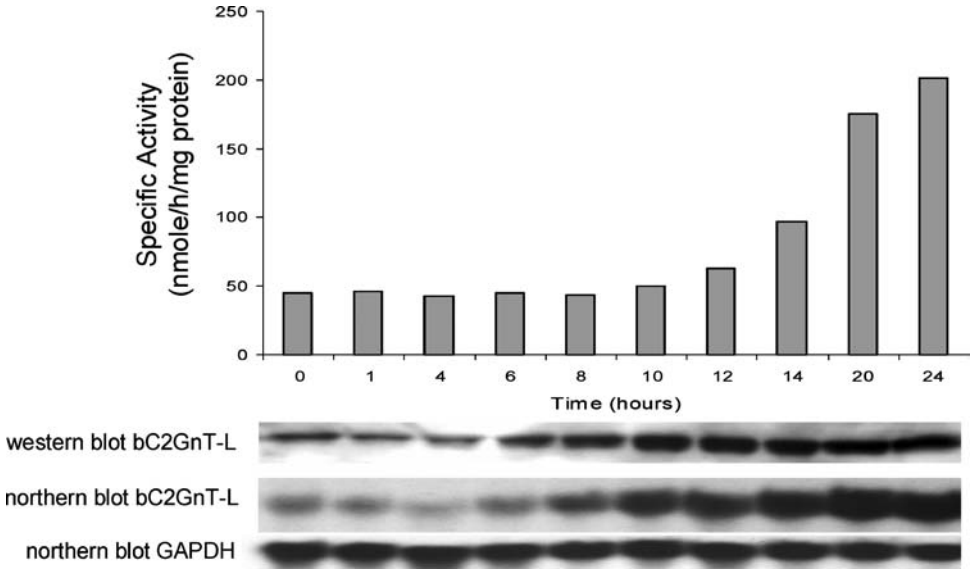

Figure 2. Time course of bC2GnT-L expression after butyrate treatment. Specific activity was measured, and western blotting and northern blotting were performed at the indicated times post treatment ($n = 3$).

the butyrate effect, which may require a coordination of several intracellular signaling steps culminating in the activation of the CMV promoter. It was noted that butyrate at concentrations greater than 2 mM was cytotoxic. Furthermore, butyrate treatment has been used to enhance the production of a recombinant glycosyltransferase in CHO cells stably transfected with the glycosyltransferase gene controlled by the CMV promoter [20].

Acetylation of histone is a well-known mechanism for gene activation [21]. Hypoacetylation of histones leaves histones in positively charged states, which promote a tight binding of histones to DNA through charge-charge interactions. Such interactions prevent access of transcription factors to the promoters and lead to inhibition of gene expression. Histone acetylation can be regulated by competitions between histone acetylase and deacetylase. Sequence analysis of the CMV promoter revealed p300 and E2F binding sites at the immediate upstream of TATA box. p300 has histone acetylase activity, and E2F can bind Retinoblastoma protein (Rb). The Rb/E2F complexes sit on the E2F binding site and blocks transcription of the target gene. In addition, strong affinity of Rb with histone deacetylases (HDACs) helps recruit HDACs to the promoter [22,23]. The recruited DHACs cause deaceylation of histones, which allows histones to bind tightly to the DNAs to prevent access of other transcription factors to the promoter, resulting in inhibition of gene expression. We propose that sodium butyrate inhibits histone deacetylase activity of the HDACs, which leave p300 to acetylate the histones unopposed. As a result, the promoter region becomes exposed to other transcription machinery, resulting in gene activation. The extent of the contribution to the activation of CMV-controlled β-galactosidase by p300 or inhibition of E2F remains to be determined.

Enhancement of the expression of pCMV-bC2GnT-L cDNA stably transfected in CHO cells treated with TSA and TSA plus butyrate

To examine if another histone deacetylase inhibitor also enhanced the expression of one of these glycogenes in these cells, TSA was used. The C2GnT activity in CHO-bC2GnT-L cells treated with TSA was increased to the same extent as that of the cells treated with sodium butyrate (Figure 3). The CHO-bC2GnT-L cells treated with sodium butyrate plus TSA exhibited slightly higher C2GnT activity than treatment

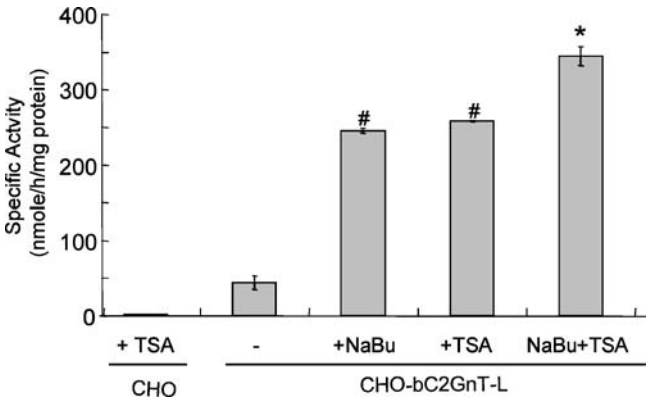

Figure 3. Effects of trichostatin A (TSA) and butyrate treatment on C2GnT activity in CHO-bC2GnT-L cells. CHO-bC2GnT-L cells ($n = 4$ for each treatment group) were treated for 24 h with 1 μM TSA, 2 mM butyrate, or both before analysis of C2GnT activity. #, $p = 0.0001$ for CHO-bC2GnT-L cells treated with TSA or NaBu vs. none; and *, $p < 0.02$ for CHO-bC2GnT-L cells treated with NaBu plus TSA vs. NaBu or TSA.

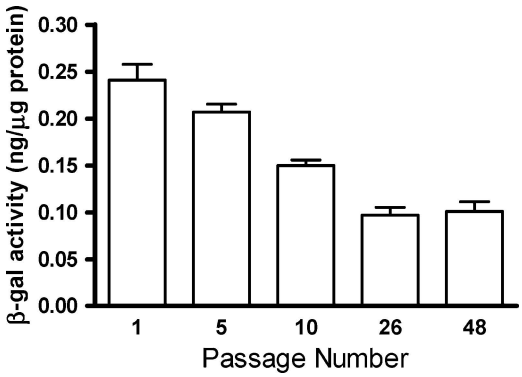

Figure 4. β-Galactosidase activity of CHO-lacZ clone 3 as a function of passage number ($n = 3$). Clone 3 was grown to confluence in T-25 flask and then split into 3 T-25 flasks. P-values of two-tail *t*-test for passage 1 versus passage 5, 10, 26, or 48 were: 0.15, 0.007, 0.001, and 0.002, respectively.

with either one. Equal enhancement of the expression of two different glycogenes (Figure 1) coupled with a slightly more enhancement in the combination treatment (Figure 3) supports the notion that both agents share major modes of action, such as inhibition of histone deacetylase. These data also suggest that each agent has its own unique mechanism for activating a silenced gene controlled by the CMV promoter. The mechanisms for transgene activation unique to each agent remains to be identified.

Sodium butyrate and TSA activate pCMV-lacZ cDNA stably transfected in CHO cells

The CHO-LacZ cells, which express β-Gal under the control of the CMV promoter, were also employed for the study because E. coli β-Gal is a very stable enzyme which can be easily stained with X-Gal. Using β-Gal as the reporter affords an opportunity for easy monitoring of the cells that express β-Gal. We have isolated 19 independent clones stably transfected with pCMVlacZ, which were divided into three groups based on specific activity. Four clones were high expressers, which expressed 0.3–0.9 ng β-Gal/μg protein. Six clones were intermediate expressers, which expressed 0.05–0.22 ng β-Gal/μg protein. Nine clones were lower expressers, which expressed lower than 0.02 ng β-Gal/μg protein. Clone 3, one of the intermediate expressers, was used for subsequent study. We found that the β-Gal in CHO-lacZ cells freshly cloned would lose about 15% of the activity after 5 passages, and 40% after 10 passages, and 60% after 26 passages and thereafter (Figure 4). The results indicated that the lacZ gene in the stably transfected CHO cells was inactivated gradually until a low basal expression level was reached. The result confirmed previous reports [3,8].

As shown in Figure 5, β-Gal activity was enhanced in CHO-LacZ cells that had been treated with butyrate or TSA. Furthermore, butyrate and TSA combination treatment showed a greater enhancement than either one alone although the difference in enhancement was not statistically significant. The result showed a similar trend as that described above (Figure 3), indicating that the phenomenon is primarily dependent on the CMV promoter and not the transgenes.

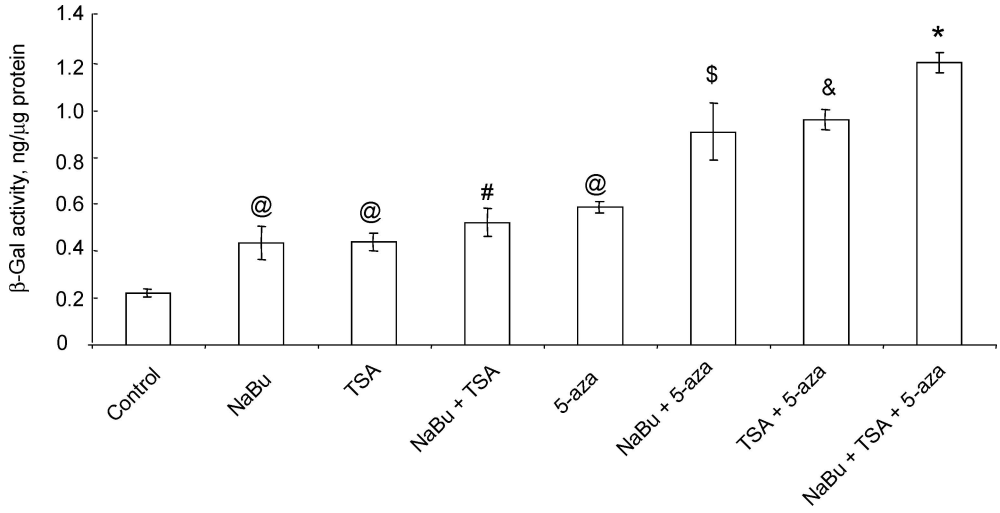

Figure 5. Effects of 5-Aza-dC, butyrate, TSA, or various combination treatments on β-Gal activity in CHO-LacZ cells. CHO-LacZ cells were treated for 24 h with 2 mM butyrate, 1 μM TSA, and 1 μg/ml 5-Aza-dC in various combinations before analysis of β-Gal activity ($n = 3$). Passage 5 of clone 3 was used for this study. @, *p* values for control vs. NaBu, TSA, or 5-aza were <0.01, <0.001, and <0.001, respectively; #, *p* values for NaBu + TSA vs. NaBu or TSA were 0.17 and 0.10, respectively; $, *p* values for NaBu + 5-aza vs. NaBu or 5-aza were 0.004 and 0.010, respectively; &, *p* values for TSA + 5-aza vs. TSA or 5-aza were 0.0001 and 0.0002, respectively; and *, *p* values for NaBu + TSA + 5-aza vs. NaBu + TSA, NaBu + 5-aza, or TSA + 5-aza were 0.0001, <0.02, and <0.003, respectively.

Table 1. Percentage distribution of blue cell populations in CHO-LacZ cells treated with butyrate or TSA

	Treatments		
Cell populations	*None*	*Butyrate*	*TSA*
	% Cell population (Mean ± SE)*		
Blue cells			
Homogeneous	7.1 ± 0.5	5.7 ± 0.6	2.9 ± 0.3#
Heterogeneous	22.3 ± 1.2	30.6 ± 1.4#	40.6 ± 2.0#
Non-Blue cells	70.6 ± 2.9	63.7 ± 0.6	56.5 ± 0.7#

*The cells were counted from 3 independent fields with an average of 130 ± 1.4 clones/field.
#$p < 0.01$ for the treated vs. the control.

To assess the efficiency of transgene expression, the % cells that expressed β-Gal were measured by X-Gal staining after these cells had been treated with either butyrate or TSA. About 30% of the untreated control cells were blue cells, and 36% and 44% of the butyrate and TSA-treated cells were stained blue, respectively (Table 1). It was noted that not all the cells derived originally from a single clone were stained blue with X-gal. The cells in these clones were classified into three groups: one stained blue homogeneously, one stained blue heterogeneously, and one not stained (Figure 6). Treatment with butyrate, TSA, or both converted cells that did not express β-Gal into ones that expressed β-Gal, reflecting activation of the CMV promoter.

Activation of LacZ gene expression in CHO cells stably transfected with pCMV-LacZ by 5-Aza-dC and its combination with either sodium butyrate, TSA, or both

Treatment of CHO cells stably transfected with pCMV-LacZ for 2 days with 5-Aza-dC, a DNA methylase inhibitor, resulted in a significant enhancement, *i.e.* 165% above control value, of β-galactosidase activity (Figure 5). Treatment of these CHO cells with 5-Aza-dC plus either sodium butyrate or TSA resulted in a similar enhancement of β-galactosidase activity (310% above control values for butyrate plus 5-Aza-dC, and 333% above control value for TSA plus 5-Aza-dC), which was more than the sum of the net enhancement of β-Gal activity by individual treatment (5-Aza-dC or NaBu = 261% above control values; TSA or 5-Aza-dC = 262% above control value). The NaBu, TSA, and 5-Aza-dC combination treatment also showed a greater enhancement of the β-Gal activity (442%) than the sum (358%) of the net enhancement of the enzyme activity by individual agents.

It is known that a transgene also can be silenced by methylation of the transduced genes [24]. This is a mechanism employed by mammalian cells to prevent the expression of foreign genes. Although not universal, histone deacetylase inhibitors, such as butyrate and TSA, also can induce genome-wide demethylation [8,9]. In addition, TSA can induce demethylation of transiently transfected genes that were ectopically methylated [10]. In our study, 5-Aza-dC plus either butyrate, TSA, or both generated a synergistic effect in the activation of a silenced β-galactosidase gene controlled by CMV promoter. This is consistent with the reported synergistic action of TSA and 5-Aza-dC combination treatment in the activation of genes silenced in cancer cells [8]. Therefore, the combination treatment of 5-Aza-dC plus either butyrate or TSA may utilize similar mechanism in the activation of the transgenes controlled by same promoter. Furthermore, the synergistic activation of CMV promoter-controlled gene expression by 5-Aza-dC plus butyrate, TSA, or both suggests that there is a cross talk between histone acetylation and DNA methylation in gene activation as previously described [8]. However, simultaneous administration of TSA and 5-Aza-dC can activate the CMV-promoter in human glioblastoma cell line U87 in a cooperative but not synergistic or additive mechanism [25]. The difference in the activation of the CMV-promoter by TSA and 5-Aza-dC combination treatment between this report [25] and our results (Figure 5) may be due to a difference in cells used in these studies. Elucidation of the full mechanisms of the activation of CMV promoter should greatly help the effort for development strategy to activate silenced CMV promoter.

Our study clearly showed that CMV promoter-controlled β-galactosidase gene stably transfected in CHO cells was inactivated over time, which can be reactivated by several agents.

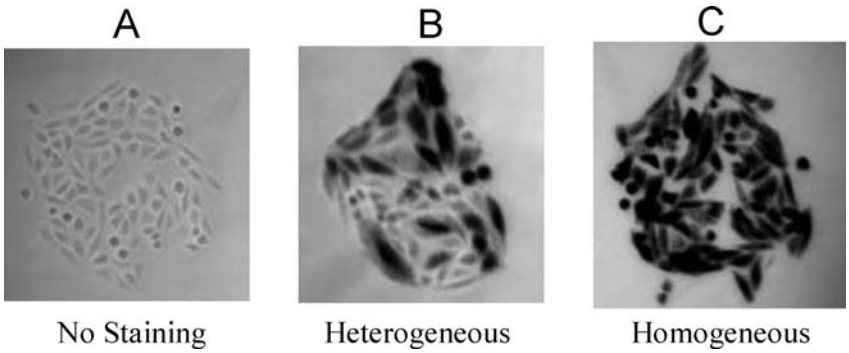

Figure 6. Representative X-Gal staining patterns of control CHO-LacZ cells and same cells treated with butyrate or TSA. (A) cells without X-gal staining; (B) heterogeneously stained cells; and (C) homogeneously stained cells.

The activation of the CMV promoter by sodium butyrate or trichostatin A is neither cell type nor transgene specific because the phenomenon occurs in different cell types and for different transgenes. However, these agents can not activate all the cells derived from a single clone. In addition, the synergistic activation of the CMV promoter-controlled transgene by combination treatment with 5-Aza-dC plus sodium butyrate, TSA, or both suggests that histone hypoacetylation and DNA methylation are the major gene inactivation mechanisms, but not the only mechanisms utilized by mammalian cells to inactivate foreign genes. Other factors such as cell cycle control or posttranslational modification of the recombinant protein, such as phosphorylation, may be affected by these two agents. Identification of these factors could help gene therapy effort by improving the expression of therapeutic genes.

Acknowledgments

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