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The regulation of the intestinal mucin MUC2 expression by short chain fatty acids: implications for epithelial protection.

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Running title: the effect of short chain fatty acids on MUC2 synthesis

ABSTRACT

Short chain fatty acids (SCFAs), fermentation products of bacteria, influence epithelial-specific gene expression. We hypothesize that SCFAs affect goblet cell-specific mucin MUC2 expression and thereby alter epithelial protection. Our aim was to study the mechanisms that regulate butyrate-mediated effects on MUC2 synthesis. Human goblet cell-like LS174T cells were treated with SCFAs, after which *MUC2* mRNA levels and stability and MUC2 protein expression were analyzed. SCFA-responsive regions and *cis*-elements within the *MUC2* promoter were identified by transfection and gel shift assays. Effects of butyrate on histone H3/H4 status at the *MUC2* promoter were established by chromatin immunoprecipitation. One mM butyrate as well as propionate induced an increase in *MUC2* mRNA levels. *MUC2* mRNA levels returned to basal levels after incubation with 5–15 mM of butyrate. Interestingly, this decrease was not due to loss of RNA stability. In contrast, at concentrations of 5–15 mM of propionate MUC2 mRNA levels remained increased. Promoter regulation studies revealed an active butyrate-responsive region at -947/-371 within the *MUC2* promoter. In this region we identified an active AP1 (c-Fos/c-Jun) *cis*-element at -818/-808 that mediates butyrate-induced activation of the promoter. Finally, MUC2 regulation by butyrate at 10–15 mM was associated with increased acetylation of histone H3 and H4 and methylation of H3 at the *MUC2* promoter. In conclusion, 1 mM of butyrate and 1–15 mM of propionate increase MUC2 expression. The effects of butyrate on MUC2 mRNA are mediated via AP-1 and acetylation/methylation of histones at the *MUC2* promoter.

Keywords: short chain fatty acids, mucin-2, necrotizing enterocolitis, histon acetylation, histon methylation, human milk feeding

INTRODUCTION

Short chain fatty acids (SCFAs) are produced by fermentation of undigested carbohydrates. SCFAs, and more specifically acetate, propionate and butyrate, are the major anions in the lumen of the large intestine. Several functions of SCFAs have been described, i.e. lowering intestinal pH, energy-source for colonocytes, stimulation of colonic blood flow, smooth muscle contraction, transepithelial chloride secretion and exertion of proliferative stimuli of colonic epithelial cells [1]. It is known that dietary fibers and SCFA have beneficial effects in inflammatory bowel disease (IBD), e.g. by inhibition of proinflammatory cytokine-induced NF κ B activation and absorption of sodium and water [2-4]. In addition, SCFAs and especially butyrate are known to influence intestinal specific gene expression, thereby influencing immune responses and oxidative and metabolic stress [5-9].

The composition of SCFAs in the intestine is determined by the composition of the microbiota, which in its turn is influenced by the diet. For example, prebiotics selectively stimulate the growth, and or, activity of bifidobacteria and thereby influence the SCFA composition [10].

Moreover, in human milk-fed infants the large bowel is generally dominated by bifidobacteria and lactic acid bacteria. The flora of formula-fed infants on the other hand, is more diverse, less stable and often contains more Bacteroides, Clostridium and Enterobacteriaceae [11-14]. This difference in the composition of the microbiota results in a different SCFA composition between human milk-fed and formula-fed infants. It is well known that the ratio between the SCFAs butyrate, propionate, and acetate differ in breast-fed infants compared to formula-fed infants (i.e. 2:6:90 in human milk fed infants versus 5:20:70 in formula fed infants) [15]. Based on the fact that more than 90% of the infants who develop necrotizing enterocolitis (NEC), which is the most common gastro-intestinal emergency in premature infants, have received formula feeding, as opposed to human milk solely, one could suggest that the production of SCFAs by bacteria and the composition of SCFAs in the intestine might play a role in the pathogenesis of NEC. Altered fecal concentrations of butyrate have also been reported in patients with ulcerative colitis (UC). In addition, a diminished capacity of the intestinal mucosa to oxidize butyrate has been reported in patients with active UC [16-18].

Both UC and NEC share the feature of an impaired intestinal barrier function. Mucins are required for the maintenance of an adequate mucus layer that covers the intestinal epithelium and thereby forms a physical barrier that protects the intestinal epithelium against toxic agents. The mucin MUC2 is the predominant mucin in the colon and MUC2 synthesis is diminished in UC [19, 20] and presumably also NEC.

It has been shown in cell line studies, experimental animal models and fresh human intestinal tissue specimens, that butyrate alters MUC2 expression in a dose dependent manner [21-25]. However, the mechanisms that are responsible for these alterations have not been studied in detail so far.

In the present study, we investigated the role of increasing concentrations of butyrate, as well as acetate and propionate on MUC2 expression in LS174T cells, a human goblet cell-like cell line. Furthermore, The effects of butyrate on MUC2 expression were respectively studied at the promoter, mRNA and protein levels. We identified butyrate-responsive regions and *cis*-elements within the MUC2 promoter and determined the effects of butyrate treatment on histone H3 and H4 status at the MUC2 promoter.

EXPERIMENTAL

Cell culture

The LS174T colonic cancer cell line was cultured in a 37 °C incubator with 10% CO₂ in Dulbecco's modified Eagle's minimal essential medium supplemented with non-essential amino acids and 10% foetal calf serum (Boehringer Mannheim) as before [26].

Cell proliferation assay and morphological alterations

LS174T cells (2×10^5) were pre-cultured in 24-well plates overnight to allow them to adhere. Subsequently cells were stimulated with physiological concentrations of butyrate (0, 1, 5, or 10 mM) (sodium butyrate, Sigma-Aldrich, Steinheim, Germany) diluted in cell culture medium for 24 h. After removal of the culture medium, cells were treated with trypsin-EDTA solution and counted. All experiments were performed in triplicate in at least three separate experiments. In addition, cell proliferation and cell death were determined using the WST-I (WST-I proliferation agent, Roche Molecular Biochemicals, Germany) and trypan blue exclusion assays, respectively. Further, butyrate-induced morphological changes were studied microscopically.

Quantitative real-time PCR

LS174T cells were seeded in 6-well plates at 0.5×10^6 cells/well. Cells were incubated 16 hours after seeding with either a low (1mM), moderate (5 mM) or high (10 and 15 mM) concentration of butyrate, acetate or propionate (Sigma-Aldrich, Steinheim, Germany). After 24 hours of stimulation, cells were lysed and harvested. Total RNA was prepared using the Nucleospin RNA II-kit from Macherey-Nagel. 1.5 µg of total RNA was used to prepare cDNA. The mRNA expression levels of MUC2 as well as the housekeeping gene GAPDH were quantified using real-time PCR (qRT-PCR) analysis (TAQman chemistry) based upon the intercalation of SYBR Green on an ABI prism 7900 HT Fast Real Time PCR system (PE Applied Biosystems) as previously described [27]. The primer combinations for MUC2 (5'-CTC CGC ATG AGT GTG AGT -3', and 5'-TAG CAG CCA CAC TTG TCT G -3') and GAPDH (5'-GTC GGA GTC AAC GGA TT -3', and 5'-AAG CTT CCC GTT CTC AG -3') were designed using OLIGO 6.22 software (Molecular Biology Insights) and purchased from Invitrogen. The effect of butyrate on MUC2 transcription and *de novo* protein synthesis was respectively studied by co-incubating cells with butyrate and either actinomycin D (0.5 µg/ml) or cycloheximide (10 µg/ml) (Sigma-Aldrich, Steinheim, Germany). qRT-PCR was performed as described above.

RNA stability assay

LS174T cells were seeded at 1×10^6 cells/well in a 6-well cluster 24h prior to the experiment. At t=0, the cells were treated with either 0 mM or 5 mM butyrate in combination with 4 µg/ml actinomycin D (Sigma-Aldrich, Steinheim, Germany). Cells were harvested after 0, 4, 6, 8, 10 and 24 h of butyrate/actinomycin D treatment. RNA isolation, cDNA synthesis and qRT-PCR were performed as mentioned above. To verify the amplification efficiency as well as the amount of mRNA present in the treated cells, a serial dilution of cDNA derived from non-treated LS174T cells was amplified in duplicate on each plate.

Cell transfections

pGL3-*MUC2* promoter constructs covering the -371/+27, -947/-1, -2096/+27 and -2627/-1 regions of *MUC2* promoter were previously described [26]. The AP-1-Luc reporter construct was a kind gift from Dr Avery (Pennsylvania State University, USA). LS174T cells were seeded at 2.0×10^5 /well in 24-well plates. Transfections and co-transfections were performed the next day by adding 0.25 μg of the pGL3 construct of interest and 0.15 μg of pHRG-B as an internal control. Transfection and co-transfection experiments were performed using Effectene[®] reagent (Qiagen) as described previously [26]. Cells were incubated with the transfection mixture for 24 h at 37 °C. Stimulation with variable dosage of butyrate was performed during 24 h. Total cell extracts were prepared using 1x passive lysis buffer (Promega), as described in the manufacturer's instruction manual. 10 μl of cell extract was used to determine luciferase activity in a Glomax luminescence counter (Promega) using the dual luciferase assay system (Promega). The luciferase activity is expressed as fold induction of the nonstimulated sample compared with that of the SCFA-stimulated samples, after correction for transfection efficiency as measured by the Renilla luciferase activity. All experiments were performed in triplicate in at least three separate experiments.

Site-directed mutagenesis

The consensus AP-1 site (ATGAGTCAGA) found in the *MUC2* promoter at -817/-808 was mutated using the QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. The sequence of the oligonucleotides used to mutate the AP-1 site are depicted in table I. The mutation was confirmed by DNA sequencing.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts from LS174T cells, untreated or treated with butyrate were prepared as described before [28], quantified using the bicinchoninic acid assay (Pierce, Perbio Science, Brebières, France) and stored at -80°C. Oligonucleotides were synthesized by MWG-Biotech (Germany), sequences are shown in Table 1. Annealed oligonucleotides were radiolabeled using T4 polynucleotide kinase (Promega) and [γ ³²P]-dATP (GE Healthcare) and purified by chromatography on a Bio-Gel P-6 column (Bio-Rad, Marnes-la-Coquette, France). Nuclear protein incubation with radiolabeled probes and competitions with unlabeled probes were as described in Perrais *et al.* [26]. For super-shift analyses, 2 μl of the antibody of interest [anti-c-fos (K-25, SC-253X) and anti-c-jun (SC-44X), Santa-Cruz laboratories, Tebu-Bio, Le-Perray-en-Yvelines, France, were added to the proteins and left for 1 h at room temperature before adding the radiolabeled probe. Electrophoresis conditions and gel processing were as described before [26].

Western Blotting

Nuclear proteins (10 μg) were separated by running a 10% SDS-polyacrylamide gel electrophoresis, followed by electrotransfer onto a 0.45 μm PVDF membrane (Millipore, Saint-Quentin en Yvelines, France). The membranes were incubated either with specific antibodies against c-fos (sc-253, 1:10,000) or c-jun (sc-44, 1:5,000) (Santa Cruz laboratories) for 1 h at room temperature, or with specific antibodies against histone H3 (anti-acetylated lysine, mono-/di-/trimethylated lysine 4) and histone H4 (anti-acetylated lysine) (Upstate #06-599 (1:10,000 dilution), #05-791 (1:10,000 dilution) and #06-598 (1:1,000 dilution), respectively for 2 h at room temperature. Secondary antibodies used were horseradish peroxidase-conjugated anti-rabbit IgGs (Pierce). For detection, blots were processed with West[®] Pico chemiluminescent substrate (Pierce) and the signal was detected by exposing the

processed blots to Hyperfilm™ ECL® (enhanced chemiluminescence; Amersham Biosciences). For Sp1 detection, the membranes were incubated 1 h at room temperature with anti-Sp1 antibody (sc-59, 1:10,000, Santa Cruz laboratories) and alkaline phosphatase-conjugated anti-goat IgGs (Promega) as secondary antibody. For detection, the membrane was incubated with Nitro Blue Tetrazolium Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies, Cergy-Pontoise, France) [29].

Immunocytochemistry

LS174T cells were grown on poly-L-Lysine coated microscope glass slides 24 h prior to butyrate treatment. Cells were treated with 0, 1, 2, 5 and 10 mM butyrate for 24h. Cells were fixed in ice-cold methanol at -20°C for 10 min and rinsed in phosphate-buffered saline (PBS). The MUC2 mucin expression was determined by immunocytochemistry. For this purpose, cells were incubated for 60 min at room temperature with the monoclonal MUC2 antibody (WE9) [30] diluted in PBS (1:200), rinsed four times with PBS, and incubated at room temperature for 60 min with the biotinylated horse-anti-mouse antibody (Vector) diluted in PBS (1:1000), followed by 1h incubation with ABC-PO complex (Vectastain Elite Kit, Vector laboratories), each component diluted 1:400 in PBS. After incubation, binding was visualized using 0.5mg/ml 3,3'-diaminobenzidine (DAB), 0.02% v/v H_2O_2 in 30mM Imidazole, 1 mM EDTA (pH7.0). The slides were counterstained with hematoxylin.

Chromatin Immunoprecipitation (ChIP)

Cells untreated or treated with butyrate (10×10^6) were fixed in 1% (v/v) formaldehyde and chromatin was sonicated and immunoprecipitated as described in Piessen *et al.* [31] with either 5 μg of specific antibodies against histone H3 (anti-acetylated lysine, mono-/di-/trimethylated lysine 4, methylated lysine 9 and trimethylated lysine 27) and histone H4 (anti-acetylated lysine) (Upstate) or with normal rabbit IgGs (Upstate) at 4°C . Immunoprecipitated chromatin (50 ng) was then used as a template for PCR using the following pairs of primers: forward primer1: 5'-TTGGCATTTCAGGCTACAGGG-3' and reverse primer1: 5' GGCTGGCAGGGGCGGTG-3', covering the -236/+24 region of *MUC2* promoter. PCR was performed using AmpliTaq Gold polymerase as described by Piessen *et al.* [31]. PCR products (15 μl) were separated on a 2% (w/v) agarose gel containing ethidium bromide run in 1X TBE buffer.

Statistical analysis

All values in this article are mean values \pm standard deviation (SD).

RESULTS

Butyrate affects cell morphology and proliferation

SCFAs are known to affect epithelial proliferation, differentiation, apoptosis, and gene expression. Butyrate is, compared to acetate and propionate, the most effective SCFA in inducing alterations in these processes. Therefore, we first analyzed the effects of various concentrations of butyrate on morphology, proliferation and apoptosis of LS174T cells. Butyrate induced marked changes in morphology, which are characterized by elongation/stretching of the cells (Fig. 1A). Furthermore, butyrate treatment inhibited the proliferation of the LS174T cells in a dose-dependent manner, as reflected by a decrease in cell number upon butyrate treatment (Fig. 1B). These data were confirmed by WST-1 cell proliferation assays (data not shown). Finally, none of the butyrate concentrations (1-10 mM) used in this study induced cell death of LS174T cells, as determined by trypan-blue exclusion assays and analysis of cell morphology.

SCFAs alter MUC2 mRNA expression

LS174T cells were stimulated with increasing concentrations, from 1 mM to 15 mM, of butyrate, propionate, or acetate to determine the effects of the different SCFAs on MUC2 mRNA expression (Fig. 2A). One mM butyrate induced a 2.5-fold increase in MUC2 mRNA levels compared to untreated cells. In contrast, stimulation with higher concentrations, i.e. 5-15 mM butyrate, did not induce an increase in MUC2 mRNA levels, as at these concentrations MUC2 mRNA levels were comparable with control levels. Similar to butyrate, 1 mM propionate induced a 2.5 fold increase in MUC2 mRNA levels. Furthermore, at 5 mM propionate, MUC2 mRNA levels increased 4.2 fold, whereas at higher concentrations MUC2 mRNA levels decreased again. Acetate treatment resulted in a dose-dependent increase in MUC2 mRNA levels as of 5 mM reaching a 2.2 fold induction at 15 mM. To determine whether the activating effect of SCFAs on MUC2 expression occurred at the transcriptional level, cells were pretreated with actinomycin D, which inhibits transcription. The results indicate that activation of MUC2 expression by 1 mM butyrate occurred at the transcriptional level, as MUC2 mRNA levels returned to basal levels when cells were pretreated with actinomycin D (Fig. 2B). This process also requires de novo protein synthesis as pretreatment of LS174T cells with cycloheximide, an inhibitor of mRNA translation, decreased MUC2 mRNA levels to basal levels as well (Fig. 2B). Similar results were obtained when cells were stimulated with propionate or acetate instead of butyrate (data not shown). Since butyrate increased MUC2 mRNA levels at low concentrations (1 mM) in contrast to no effect at moderate (5 mM) and high (10 and 15 mM) concentrations, we studied whether this decrease was due to a decrease in MUC2 RNA stability. For that we pre-treated cells with actinomycin D over a 24h period of time, and then incubated cells with 5 mM butyrate before measuring MUC2 mRNA amount by qRT-PCR. The results show no differences in MUC2 mRNA stability between butyrate-stimulated and non-stimulated cells (Fig. 2C).

Effect of butyrate on MUC2 protein expression

To determine whether butyrate also induced an increase in MUC2 protein expression in LS174T cells, immunocytochemistry was performed with an antibody specific for MUC2. In non-stimulated cells MUC2 staining was hardly visible (Fig. 3). Stimulation with 1 mM of butyrate clearly shows an increase in MUC2 staining. This effect was even more pronounced in cells stimulated with 2 mM of butyrate (Fig. 3).

Identification of butyrate responsive regions in the MUC2 promoter

Transfections with *MUC2* promoter constructs were performed to identify butyrate-responsive regions. The *MUC2* promoter constructs used are indicated in Figure 4A. Stimulation of LS174T cells with low (0.5-2 mM) concentrations of butyrate demonstrated a dose-dependent increase in luciferase-activity after transfection with each of the promoter construct used (Fig. 4B). The highest transactivation was seen using *MUC2* promoter construct -947/-1 indicating a possible butyrate-responsive element within the -947/-372 region. Analysis of the *MUC2* promoter sequence indeed revealed the presence of a consensus putative binding site (ATGAGTCAGA) for the transcription factor AP-1 at -817/-808, a transcription factor known to mediate butyrate-induced transcriptional effects. To determine whether this putative AP-1 binding site was responsible for the butyrate-induced *MUC2* promoter transactivation, specific nucleotides within the sequence were mutated (Table 1). The mutation resulted in a 50% reduction of the butyrate-induced *MUC2* transactivation (Fig. 5A). Activation of AP-1 by butyrate in LS174T cells was confirmed by treating AP-1-Luc-transfected cells with butyrate. The stimulation was dose-dependent with a maximal 13.3-fold induction at 2 mM butyrate (Fig. 5B).

C-fos and c-jun bind to the AP-1 element in MUC2 promoter

As the transcription factors c-fos and c-jun are known to bind as a complex to AP-1 binding elements within promoters, EMSAs were carried out to show the binding of these two transcription factors to the AP-1 element found at -817/-808. When incubated with nuclear extract from untreated and butyrate-stimulated (1 mM and 10 mM) LS174T cells, the radiolabeled probe T282 (containing the putative AP-1 binding site, see table I) produced one retarded band (Fig.6, lane 2). Specificity of the protein-DNA complex was confirmed by strong decrease of the shifted band when unlabeled competition was performed with a 50 times excess of unlabeled T282 probe (lane 3), whereas competition with a 50 times excess of unlabeled mutated T282 probe (lane 4) did not affect the shifted band. Involvement of c-jun and c-fos in the complex formation was then proven in supershift experiments carried out with antibodies specific for c-jun (lane 5) and c-fos (lane 6), respectively. Addition of the two antibodies indeed resulted in a supershift that was observed both in untreated cells and butyrate-stimulated cells. This was well-correlated with the amount of c-fos and c-jun found in the cells (Fig. 7). Altogether, this suggests that the decreased *MUC2* mRNA levels after stimulation of cells with 5 mM or 10 mM butyrate compared to 1 mM butyrate stimulation (see Fig. 2), are not caused by a decreased binding capacity of AP-1 (i.e. the c-fos/c-jun complex) to its *cis*-element within the *MUC2* promoter.

Butyrate alters histone status at the MUC2 promoter in a dose-dependent manner

Since butyrate is known to affect histone deacetylase (HDAC) activity, and that *MUC2* promoter is known to be regulated by HDAC [32], we hypothesized that histone status at *MUC2* promoter may be involved in *MUC2* regulation by butyrate. To determine whether alterations in *MUC2* expression correlated to changes in histone acetylation and/or methylation we first examined the effect of butyrate on the levels of acetylated H3 and H4 histones as well as m/d/t methylated H3K4 in LS174T cells by Western blotting (Fig. 7). Acetylated histone H3 and H4 and mono-/di-/tri-methylated H3 on lysine (K) 4, which correlate with activation of transcription, were strongly increased after stimulation with both moderate (5 mM) and high (10 mM) concentrations of butyrate.

To establish the effects of butyrate on histone H3 and H4 status at the *MUC2* promoter, ChIP assays were performed with chromatin from non-stimulated and butyrate-stimulated LS174T cells (Fig. 8). In untreated and 1 mM butyrate-treated cells, *MUC2* promoter covering the -236/+24 region was

mainly associated with mono-/di-/tri-methylated K4H3 as well as to a lower extent with acetylated histone H3 and H4, which correlate with activation of transcription. At 5 mM butyrate this status of chromatin activation was confirmed with a stronger association with acetylated H3. At these two concentrations we also observed an increase of 3mK27H3, which is usually indicative of transcription inhibition. At 10 mM butyrate, histone modifications at the MUC2 promoter were characterized by modifications inducing active chromatin (AcH3, AcH4 and m/d/tK4H3) and usually indicative of inactive chromatin (mK9H3).

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DISCUSSION

In the present study we analyzed the effect of SCFAs on epithelial cell morphology, proliferation, and, as marker for epithelial protection, MUC2 expression. Moreover, we identified the mechanisms responsible for the butyrate-induced changes in MUC2 expression. By studying these parameters in conjunction we aimed to gain more insight in the effects of SCFAs on epithelial protection.

Our study revealed that butyrate altered the morphology of LS174T cells by inducing cell elongation/stretching. This suggests that butyrate affects LS174T cell differentiation. Additionally, butyrate caused a dose-dependent decrease in cell number. As this SCFA did not induce apoptosis at the concentrations used in this study, we conclude that butyrate inhibits epithelial proliferation. Several *in vitro* studies support our finding that butyrate inhibits proliferation [33-37]. For example, butyrate inhibited the proliferation of non-confluent and sub-confluent HT-29 cells in a dose-dependent manner (1-8 mM)[36]. Furthermore, *Siavoshian et al.* demonstrated that the mechanism through which butyrate inhibits proliferation in HT-29 cells is exerted via the induction of cyclin D3, an inhibitor of cell cycle progression and p21, a stimulator of cell differentiation [33].

Next, we studied the effect of SCFAs on MUC2 synthesis in the LS174T cell line. Butyrate, propionate, as well as acetate, were able to increase MUC2 mRNA synthesis. Specifically, butyrate increased MUC2 mRNA levels at low concentrations and had no effect at moderate and high concentrations. Both low and moderate propionate concentrations increased MUC2 mRNA levels, whereas at higher concentrations, MUC2 mRNA levels were still increased but to a lesser extent. Finally, a dose-dependent increase in MUC2 mRNA levels was seen after stimulation with acetate, with the smallest increase at low concentrations and highest increase at high concentrations.

Of the SCFAs studied in this paper, only the effects of butyrate on mucin expression have been described extensively. Hatayama *et al.* also showed that concentrations of 1-2 mM butyrate increased MUC2 expression in LS174T cells [22]. Barcelo *et al.* [23] demonstrated a significant discharge of mucins at concentrations of 5 mM of butyrate, while increasing the concentration to 100 mM decreased this mucus response in mice. Highly relevant, *ex vivo* stimulation of macroscopically normal fresh colon tissue with 0.05-1 mM butyrate stimulates MUC2 synthesis, whereas at stimulation with 10 mM butyrate MUC2 synthesis levels returned to basal levels [24]. These studies correlate with our data with respect to the effects of butyrate on MUC2 expression (i.e. increase in MUC2 at low concentration and no effect at high concentrations).

Despite previous studies showing induction of MUC2 by butyrate, no precise analysis of the molecular mechanisms has been performed [21-25]. Since butyrate is known to mediate its effects via the AP-1 transcription factor, and because we found a putative consensus binding site (ATGAGTCAGA) for AP-1 at -817/-808 in the MUC2 promoter, we studied its regulation by AP-1. AP-1 is a multiprotein complex, composed of the products of c-Jun and c-Fos proto-oncogenes. Growth factors, neurotransmitters, polypeptide hormones, bacterial and viral infections as well as a variety of physical and chemical stresses, employ AP-1 to translate external stimuli, both into short-term and long-term changes of gene expression. Interestingly, we found that butyrate was able to activate an AP-1 reporter construct and to induce c-Fos and c-Jun protein expression in the LS174T cell line, indicating that butyrate-induced MUC2 transcription might occur via AP-1 binding to the MUC2 promoter. That is what we indeed demonstrated by mutating the consensus AP-1 binding site, which abolished both binding of AP-1 and inhibited butyrate-induced MUC2 activation. As butyrate only increased MUC2 mRNA and protein levels at low concentrations (1-2 mM), but not at high concentrations (5-10 mM), this suggested that activation of the MUC2 promoter, and up-regulation of MUC2 RNA and protein levels, at low concentration of butyrate was, at least partly, regulated by AP-1.

Since butyrate is a well-known HDAC inhibitor, butyrate-induced alterations in gene expression can also reflect changes in histone modification status and chromatin marks. To assess whether the increase of *MUC2* expression following butyrate treatment was associated with chromatin status, we performed ChIP experiments. As expected, our data indicate that butyrate treatment is associated with dose-dependent increased of both global histone acetylation levels and histone H3 and H4 acetylation at *MUC2* promoter region. Since cross talk between histone post-translational modifications are important in establishing the histone code, increased mono-/di-/trimethylation of K4H3 observed at *MUC2* promoter after butyrate treatment may be directly linked to increased histone H3 acetylation, as previously shown by Nightingale *et al.* [38]. For a long time methylation of K9H3 has been considered as a chromatin mark associated with heterochromatin and gene silencing. However, a recent study showed that higher H3K9 monomethylation levels are detected in active promoters surrounding gene transcription start sites, suggesting that this modification may be associated with transcription activation [39]. These data are concordant with our present results, showing that in LS174T cells, the proximal region of *MUC2* promoter is associated with monomethylation of K9H3. However, to our knowledge, this is the first time that a positive effect of high concentrations of butyrate on K9H3 methylation is shown. Strikingly, we found that treatment with low concentration of butyrate induced an increase of H3K27 trimethylation at *MUC2* promoter, which therefore adopted a bivalent chromatin pattern. This bivalent profile has already been described for embryonic stem cell genes as well as DNA-hypermethylated genes which were reexpressed by demethylation [40]. We previously showed that *MUC2* is regulated by a complex combination of DNA (de)methylation and establishment of a (de)repressive histone code [41]. The changes of global epigenetic profile stated at *MUC2* promoter may thus be partly responsible for the increase in *MUC2* expression level induced by low concentration of butyrate.

Surprisingly, treatment with moderate and high concentrations of butyrate, yet associated with active chromatin marks at *MUC2* promoter, did not induce increased *MUC2* expression. Dual effects of HDAC inhibitors on gene expression have already been shown for numerous genes [43], including mucin genes. In particular, Augenlicht *et al.* [44] demonstrated that cell treatment with butyrate induce an inhibition of *MUC2* expression, correlated with repression of secretory functions of colonic cells. This repression may be due to changes in histone modification patterns, since trichostatin A, another HDAC inhibitor, has the same inhibiting effect on *MUC2* expression in LS174T cells [42]. However, our results clearly show that *MUC2* promoter is associated with active chromatin marks at high concentrations of butyrate. Therefore, the dual effect observed at high concentrations, is most likely due to dramatic butyrate-induced changes in the global chromatin landscape [42], rather than direct histone modifications at *MUC2* promoter. Numerous studies showing that expression and post-translational modifications of factors known to positively or negatively regulate *MUC2* transcription (including, among others, Sp3, CDX-2 or p53 transcription factors [43-45]) is dramatically affected by butyrate, support this hypothesis.

In conjunction, we showed that butyrate stimulates *MUC2* expression at low concentrations, but has no effect on *MUC2* expression at moderate and high concentrations. We therefore hypothesize that low concentrations of butyrate could have a protective effect on intestinal barrier function by increasing mucus production, while moderate to high concentrations may decrease intestinal barrier function by decreasing *MUC2* production. This effect might partially explain the difference in incidence of NEC between the formula-fed and human milk-fed newborn infants. Manipulation of the SCFA profile can be established by influencing the composition of microbiota, for instance by treatment with prebiotics, probiotics and or human milk. This approach seems to be promising in the treatment of IBD and NEC.

In summary, our *in vitro* data indicate that low concentrations of butyrate stimulate MUC2 mucin expression, which *in vivo* would lead to an increased intestinal epithelial barrier function. On the contrary, high concentrations of butyrate decrease MUC2 expression which might diminish intestinal barrier function.

Moreover, identification of AP-1 and histone modifications as mechanisms involved in MUC2 regulation by butyrate may represent pathways to target prevention of IBD and NEC by influencing SCFA production by the intestinal flora.

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FIGURE LEGENDS

Figure 1. Butyrate affects cell morphology and proliferation

(A) LS174T cells stimulated with 1, 5 or 10 mM butyrate (right panel) demonstrate stretching/elongation and flattening compared to untreated cells (left panel). (B) Cell counts before and after butyrate stimulation show a dose-dependent decrease in cell number after butyrate stimulation.

Figure 2. SCFAs alter MUC2 mRNA expression

(A) MUC2 mRNA fold activation in LS174T cells upon stimulation with SCFAs (butyrate, propionate and acetate) compared to untreated cells (ref.). (B) To determine whether butyrate stimulation influences MUC2 synthesis on transcriptional or translational level, LS174T cells were stimulated with butyrate (B) (1 mM) in the presence of cycloheximide (CHX) or actinomycin D (ActD). (C) Stability of MUC2 mRNA (left panel) and GAPDH mRNA (right panel) over time was measured by RT-PCR. LS174T cells were stimulated with butyrate (5 mM) after which mRNA synthesis was ceased through addition of actinomycin D. Relative mRNA expression was determined at the given time points (0, 4, 6, 8, 10 and 24 hours after addition of actinomycin D). All results represent means \pm SD obtained in triplicate in three separate experiments

Figure 3. Effect of butyrate on MUC2 protein expression

MUC2 apomucin expression by immunocytochemistry in non-stimulated (0 mM) and butyrate (1 and 2 mM) stimulated LS174T cells. (Magnification x40)

Figure 4. Identification of butyrate responsive regions in the MUC2 promoter

Schematic representation of MUC2 promoter and the different constructs used to study MUC2 promoter activity (A). Numbering refers to transcription initiation site designated +1. (B) Transfected cells were stimulated with butyrate as described in experimental procedures. Results are expressed as fold activity in the butyrate-stimulated cells compared to non-stimulated cells. Results represent means \pm SD obtained in triplicate in three separate experiments.

Figure 5. The effect of site directed mutagenesis on MUC2 promoter activation by butyrate

(A) Relative luciferase activity diagram showing the effect of site-directed mutagenesis on MUC2 promoter activation by butyrate. Cells were transfected either with the wild-type -947/-1 construct (WT, white bars) or with the AP-1 mutated construct (mut, grey bars) before being stimulated with butyrate as described in experimental procedures. (B) Luciferase activity in cells transfected with AP-1-Luc reporter construct and stimulated with butyrate. Results are expressed as fold activity of the butyrate-stimulated compared to non-stimulated cells. Results represent means \pm SD obtained in triplicate in three separate experiments.

Figure 6. C-fos and c-jun bind to the AP-1 element in MUC2 promoter

Identification of an AP-1 *cis*-element in MUC2 promoter by EMSA. Nuclear extracts from untreated cells or cells treated with either 1 or 10 mM butyrate. Radiolabeled probe T282 alone (lane 1), radiolabeled T282 with nuclear extract (lane 2), cold competition with 50x excess of unlabeled wt T282 probe (lane 3), cold competition with 50x excess of unlabeled mutated T282 (lane 4). Supershift analysis was performed by preincubating the nuclear extract with 1 μ l of anti-c-jun (lane 5) and anti-c-fos (lane 6) antibodies, respectively. Arrows and stars indicate positions of the shifted and supershifted (ss) protein-DNA complexes.

Figure 7. Expression of acetylated histones upon butyrate stimulation

Study of the expression of acetylated histones H3 and H4, m/d/t methylated H3K4, c-Fos, c-Jun and Sp1 in untreated (-) and butyrate-treated (1, 5 and 10 mM) LS174T cells by western-blotting.

Figure 8. Butyrate alters histone status at the MUC2 promoter in a dose-dependent manner

Study of the histone status at the MUC2 promoter by ChIP. Acetylated histone H3 (AcH3, lane 1), acetylated histone H4 (AcH4, lane 2), and m/d/tK4H3 (lane 5) are representative of activation of transcription whereas histone H3 methylated on lysine 9 (mK9H3, lane 3) and histone H3 trimethylated on lysine 27 (3mK27H3, lane 4) are representative of inhibition of transcription. Lane 6: negative control with IgGs. Lane 7: Input.

TABLE

Table I. Sequences of the sense oligonucleotides used for site-directed mutagenesis and EMSA. AP-1 consensus sequence is italicized. Mutated nucleotides are bold and underlined. Antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotide to produce double-stranded DNA.

Mutations	Sequence 5'-3'
<i>Site directed mutagenesis</i>	
WT 114 (-830/-795)	CAG GAT CCC CAC <i>CAT GAG TCA</i> <i>GAG GTA GTT CTG GGG</i>
Mutated T114	CAG GAT CCC CAC <u>CAG</u> GAG <u>CCA</u> <i>GAG GTA GTT CTG GGG</i>
<i>EMSA</i>	
T282 (-822/-804)	CCA CCA TGA GTC AGA GGT A
Mutated T282	CCA CCA <u>TTA</u> GTG AGA GGT A

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FIGURES

Figure 1

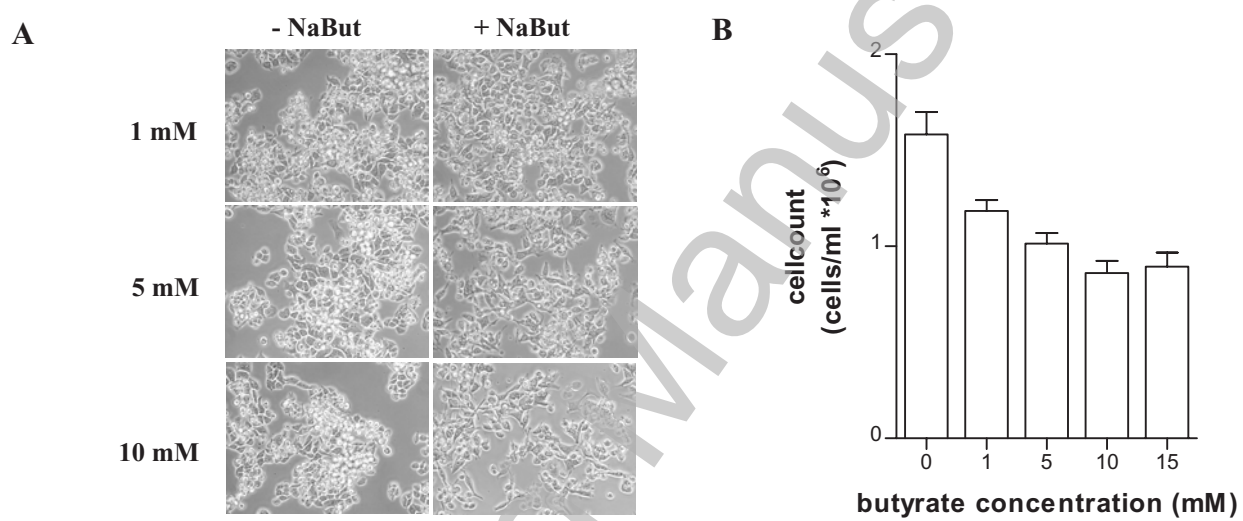
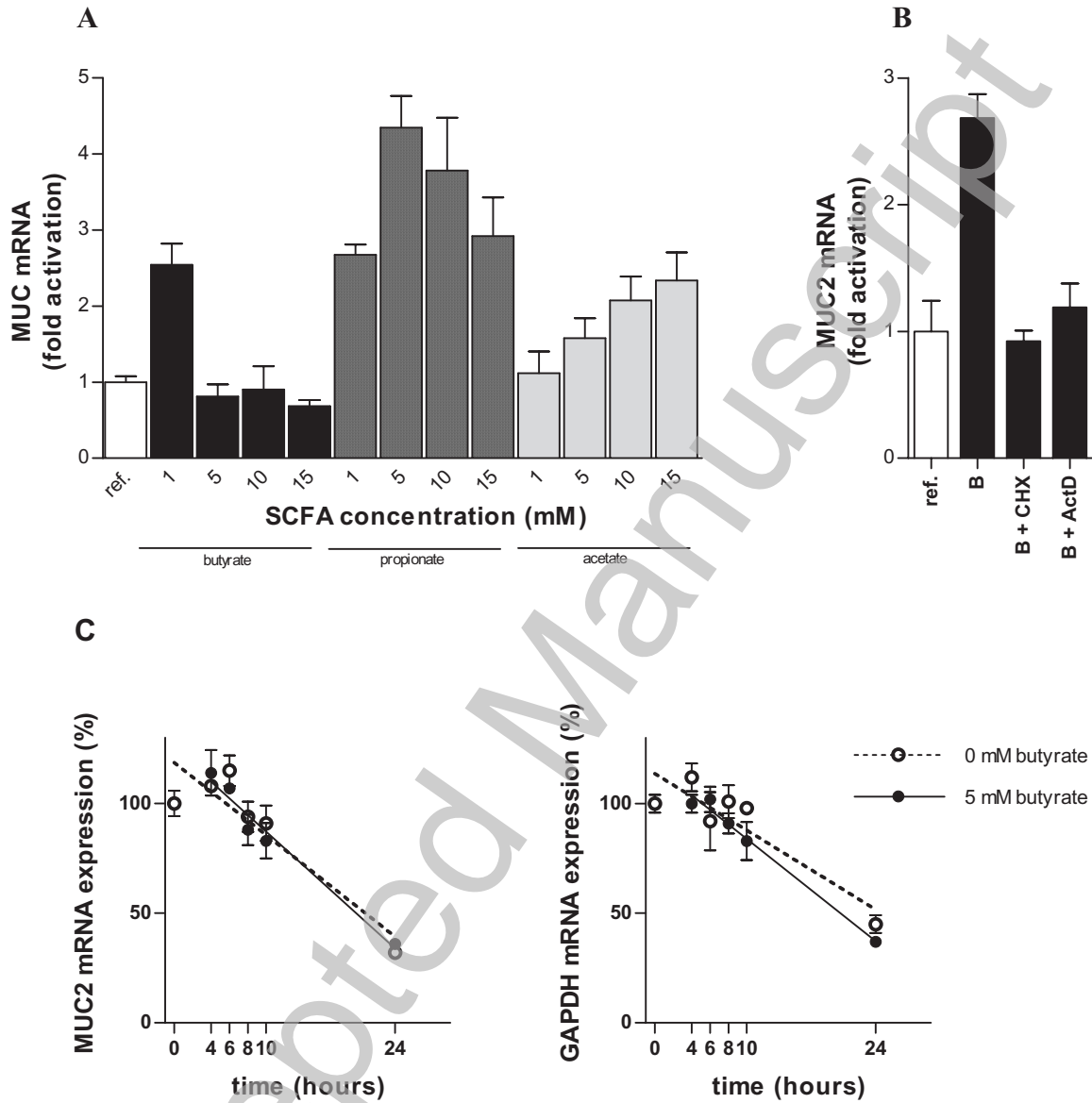


Figure 2



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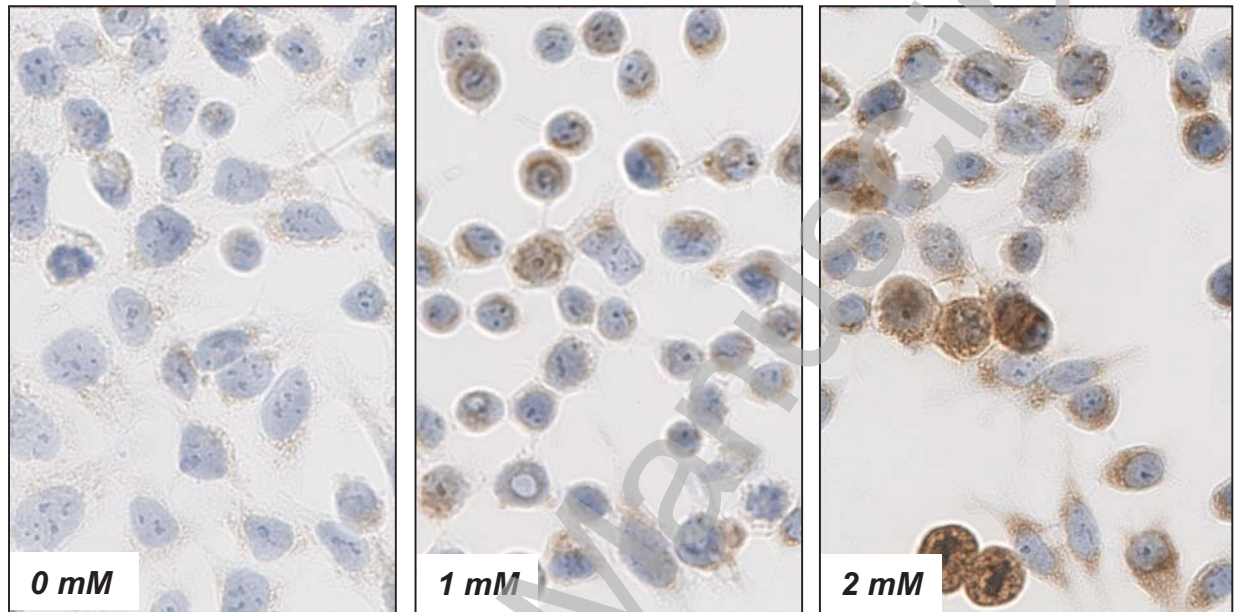


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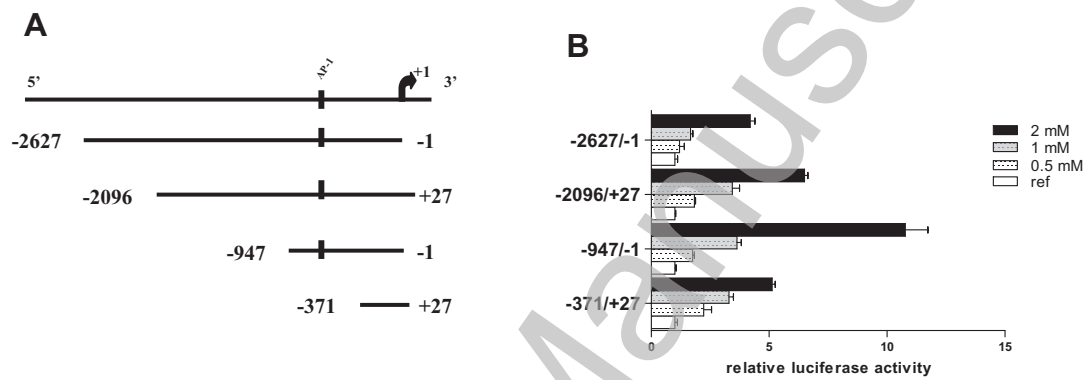


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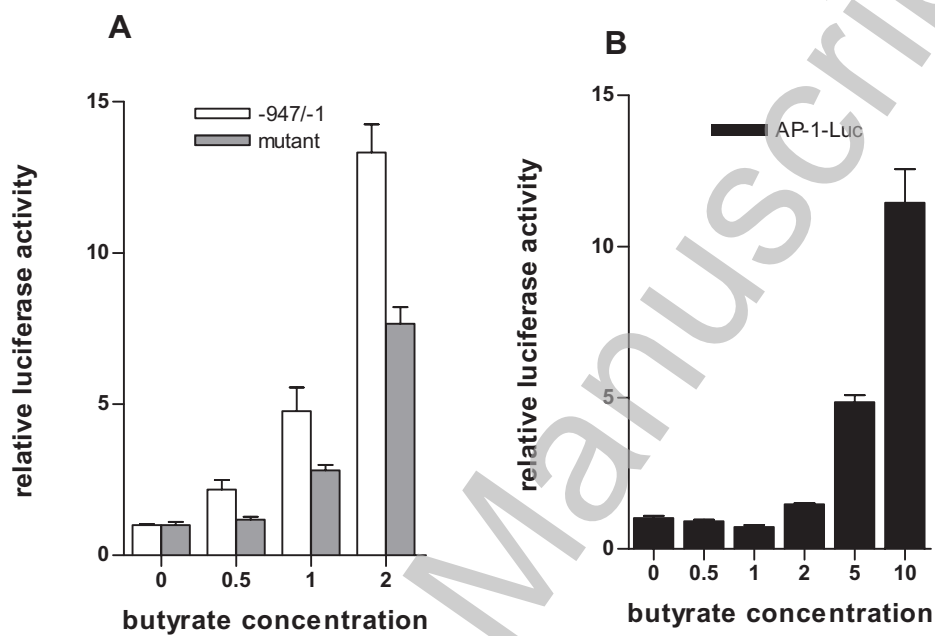
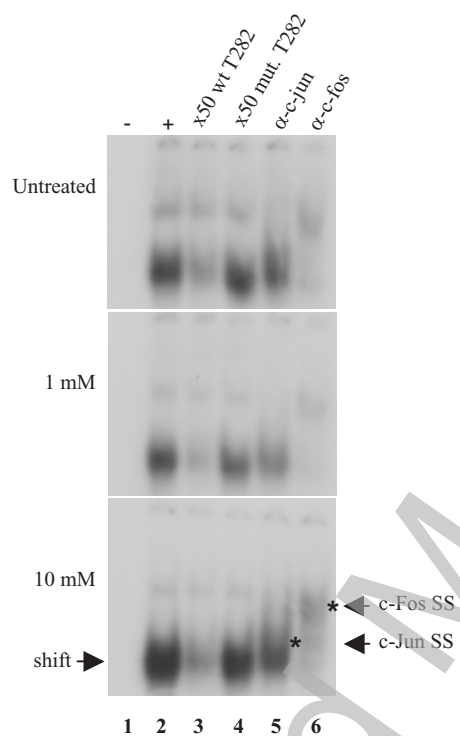
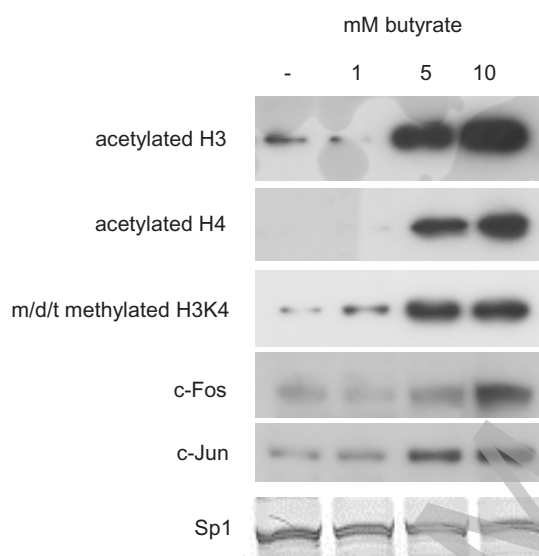


Figure 6



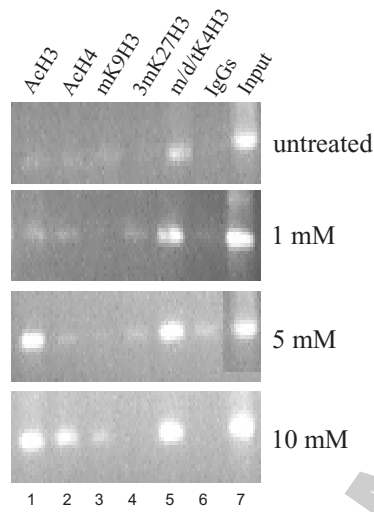
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Figure 7



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Figure 8



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