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Activation of the γ-glutamyltransferase promoter 2 in the rat colon carcinoma cell line CC531 by histone deacetylase inhibitors is mediated through the Sp1 binding motif

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Abstract

The single-copy gene for rat γ-glutamyltransferase (GGT) encodes at least seven distinct mRNAs that differ in their 5′-untranslated regions only. Tissue- and developmental-specific expression of GGT is partly achieved by the presence of many transcription factor-binding sites in the promoters of this gene. In an earlier study we found that GGT mRNAs II and IV levels were increased upon butyrate-induced differentiation of the rat colon carcinoma cell line CC531. The mechanism for this butyrate-induced upregulation remains unknown, but may result from altered promoter activity as butyrate is a known histone deacetylase inhibitor. In the present study, we show by transient transfection studies that butyrate enhanced the expression of the *luciferase* reporter gene driven by the rat GGT promoter 2 (P2). Trichostatine A (TSA), another histone deacetylase inhibitor, also enhanced transcription from this promoter. The role of the transcription factor site Sp1 in butyrate- or TSA-induced activation of the GGT P2 was examined as Sp1 has been previously shown to play a central role in the transcriptional activation of other genes during butyrate and TSA stimulation. A triple sequence-motif of this isolated Sp1 site linked to a minimal promoter was able to mediate butyrate- and TSA-induced expression of the *luciferase* reporter gene, while no effect was measured using the minimal promoter alone. Deleting the Sp1 site in the context of the rat GGT P2 strongly reduced the basal transcription activity and abrogated butyrate- and TSA-induced activation of the mutated promoter. These results suggest that butyrate- or TSA-induced activation of the rat GGT P2 can be mediated by a Sp1 binding motif.

Keywords: γ-Glutamyltransferase; Glutathione; Promoter; Histone deacetylase inhibitors; Butyrate; Sp1 transcription factor site

1. Introduction

γ-Glutamyltransferase (GGT, EC 2.3.2.2, (5-L-glutamyl)-peptide/aminoacid-5-glutamyltransferase) is involved in the metabolism of glutathione (GSH) and other γ-glutamyl compounds. GSH is an important antioxidant that protects cells against free radical damage by providing reducing equivalents for several anti-oxidising enzymes [1]. The enzyme GGT catalyses the first step in the extracellular hydrolysis of GSH and is frequently upregulated during GSH depletion [2,3]. GGT may therefore provide cells with extra cysteine for renewed synthesis of GSH and can also increase the growth of tumours *in vivo* [4].

The rat GGT protein is encoded by a single-copy gene that covers approximately 34 kilo-base-pairs (kb) and includes 12 exons encompassing a 12 kb genome fragment [5–7]. Five distinct promoters (P1–P5) can control the transcription of the gene. Transcription from these different promoters generates at least seven distinct mRNAs that differ in their 5′-untranslated regions. Both promoters P4 and P5 yield two primary transcripts due to alternative splicing, while transcription from the other promoters generates single transcripts [8–10]. Each promoter seems to be activated in a cell-specific manner, which results in cell-specific transcription of GGT mRNAs. Cell-specific expression of the GGT gene is obtained through the combination of several regulatory elements which bind cell-specific transcription factors [8,10–13].

Several stimuli, including butyrate, menadione and cis-platin have been reported to induce the expression of GGT [3,14]. However, little is known about the regulatory

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Abbreviations: CC531 cells, rat colon carcinoma cells; GGT, γ-glutamyltransferase; GSH, glutathione; TSA, trichostatine A; P1–P5, GGT promoters; HDAC, histone deacetylase; HAT, histone acetyltransferase.

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elements in the GGT promoters that mediate the induction by these stimuli. The rat GGT P3 promoter is TATA-less and dependent upon an initiator element for initiation of transcription [11,15]. Expression from this promoter in differentiated hepatoma cell lines has been associated with the factors NF-I and NF-Y [11]. Rat GGT P4 lacks a TATA-sequence or known initiator. This promoter is active in epididymis and biliary cells [16,17] and a PEA3 binding sequence probably responds to testicular factors in order to initiate transcription [18]. The fifth promoter, GGT P5, requires the binding of activator protein 1 (AP-1) and HNF-3 for transcription. Additionally, NFκB, AP-1, activator protein 2 (AP-2) and androgen receptor binding sites are present, but their functional role has not been examined so far.

Sequencing the −505 to +84 bp region of the rat GGT P2 revealed the presence of a TAAT motif 25 bp upstream of the transcription initiation site, putative CAAT motifs, and putative bindings sites for the transcription factors AP-1, AP-2, SRF, Sp1, NFκB, androgen acceptor, glucocorticoid receptor (GRE), and heat shock protein 70 [8,11,12] (Fig. 1). The contribution of these individual elements to the basal transcriptional activity of the GGT P2 has not been studied, but transient transfections studies with GGT P2 revealed that this promoter, coupled to a chloroamphenicol acetyl transferase (CAT) reporter gene, was able to drive a significant activity. Also, the region between −528 and −322 mediated activation of promoter activity. Deleting the sequences −322 to −114 increased promoter activity, suggesting that this region exerts a negative effect on the activity of the promoter [8].

Our previous studies demonstrated that GGT activity was increased in butyrate-treated rat colon carcinoma cells (CC531 cells). Only GGT mRNA II and IV levels were upregulated after butyrate treatment [14]. Several studies on regulation of genes by the HDAC inhibitor butyrate have shown that Sp1 sites can mediate butyrate-induced

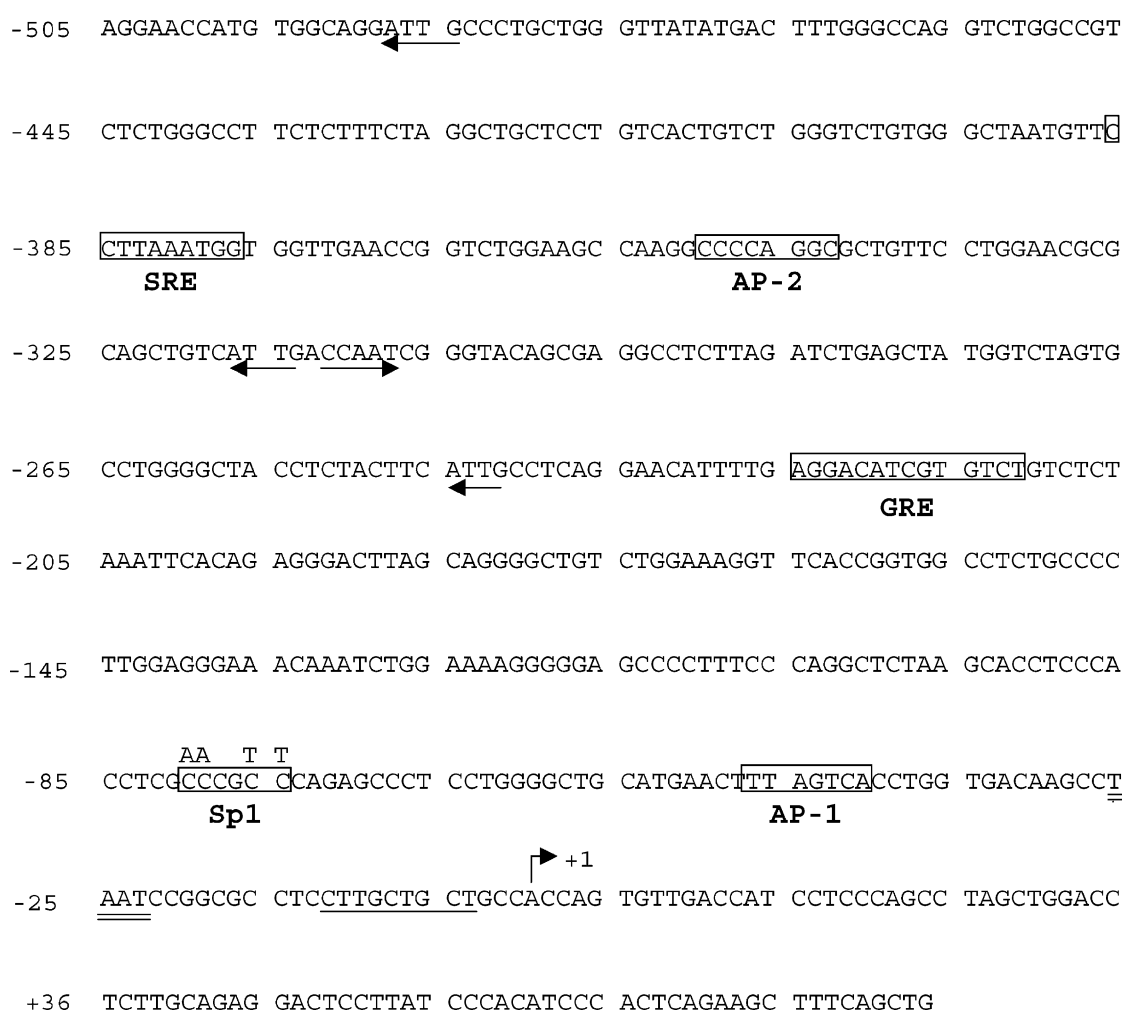

Fig. 1. Sequence and functional organisation of the rat GGT P2. The position of the major transcription site, indicated by an arrowhead, is designated as +1. Sequences downstream of the major transcription initiation sites are referred to as "+" and those upstream as "−". Putative response elements for the GRE, the serum response factor (SRE), AP-1 and AP-2, and Sp1 are enboxed. The CAAT elements are underlined with arrows. The consensus sequence flanking the cap site is underlined once, while the TAAT motif is underlined twice [8]. The mutated nucleotides in the Sp1 motif are shown above the box.

stimulation of, e.g. the *WAF1/Cip1* and *gal-1* gene [19,20] as well as inhibition of, e.g. the *ITF* gene [21]. TSA has also been found to activate the transcription of certain genes (e.g. *p21*, *dhfr*) through the Sp1 site [22,23]. These observations prompted us to examine whether butyrate could alter the activity of GGT P2 and whether the putative Sp1 motif could be involved in regulation of GGT P2 by butyrate. Our results suggest that the Sp1 site in GGT P2 is crucial for basal activity and that Sp1 probably is involved in mediating butyrate-induced activation of this promoter in the CC531 cells.

2. Materials and methods

2.1. Cells

The rat colon carcinoma cell line CC531, originally developed after chemical carcinogenesis [24], was cultured in standard RPMI 1640 medium supplemented with 5% foetal calf serum (BioWhittaker). Cells were maintained in a humidified 5% CO_2 atmosphere at 37°.

2.2. Plasmids

The luciferase reporter plasmid pGL-II-luc with the rat GGT P2 sequence −505 to +84 was generated by subcloning a fragment from pGEM 3-Z (Promega) [25]. For this purpose, a fragment spanning these sequences was amplified using the oligonucleotides: 5′-GGGGTACCAGGAACCATGTGGCAGGA-3′ and 5′-CAGCTGAAAGCTTCTGAG-3′. These oligonucleotides contain restriction sites underlined for *Kpn* I and *Hind* III, respectively. The amplified product was subsequently cut with these enzymes and cloned in the corresponding sites of the luciferase reporter plasmid pGL3 (Promega).

The plasmid pGL-II mutSp1-luc was generated by site-directed mutagenesis using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). For the creation of the Sp1 mutation in GGT P2, the complementary oligonucleotides 5′-GCACCTCCCACCTCG**AACGTT**CAGAGCCCTCCTGGG-3′ and 5′-CCCAGGAGGGCTCTG**AACGTT**CGAGGTGGGAGGTGC-3′ were used. This mutation (underlined) introduced an *Acl* I restriction site (shown in bold) used for analysis. The mutation of the Sp1 site was verified by automated sequencing (ABIprism 377, Perkin Elmer). The plasmid pSp1-luc, containing three adjacent Sp1 motifs of GGT P2 was generated by annealing the complementary oligonucleotides 5′-CTACCCGCCTAATCCCGCCTTACCCGCCTATTA-3′ and 5′-GATCTAATAGGCGGGTTAAGGCGGGATTAGGCGGGTAGGTAC-3′, and subsequent cloning in the *Kpn* I and *Bgl* II sites of the enhancer-less luciferase reporter vector pTAL-luc (Clontech Laboratories Inc.). The plasmid was verified by automated sequencing (ABIprism 377, Applied Biosystems).

2.3. Transient transfection studies

For transient transfection studies, 3×10^5 CC531 cells were seeded per well in six well cell culture plates (Nunc Brand Products) one day before transfection. This resulted in 60–70% confluency at the time of transfection. The next day, cells were transfected with 1.5 μg luciferase reporter plasmid using Lipofectamine2000 (Invitrogen). The transfected cells were incubated for 24 hr at 37° before stimulation. Medium was replaced by fresh medium with or without stimulants. Transfected cells were stimulated with 2 mM sodium butyrate (Sigma Aldrich) or 0.75 μM trichostatine A (TSA; Sigma Aldrich) for either 6, 12 or 24 hr.

2.4. Luciferase activity

The transfected cells were harvested in 500 μL lysis buffer (Tropix, PE Biosystems) containing 1 mM DTT (Invitrogen). Luciferase activity was measured in 20 μL cell extract using the Luminoscan RT luminometer (Labsystems Oy). The luciferase activity in each cell extract was adjusted to the total protein concentration. The Bio-Rad DC Protein Assay kit was applied to measure protein concentrations (Bio-Rad Laboratories).

2.5. RNA isolation and GGT mRNA II quantitation

Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen), according to the recommendations from the manufacturer. For RT–PCR, 1 μg of RNA was used together with RNAsine (Promega), oligodT (Invitrogen) and SuperscriptII (Invitrogen). For PCR, the cDNA was amplified with Dynazyme (Finnzyme Oy) and gene specific primers for GGT mRNA II [10] and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the primer-dropping method [26]. The PCR products were visualised by ethidium bromide staining after electrophoresis and the gels were scanned using Bio-Rad Multianalyst TM/PC (Bio-Rad Laboratories).

2.6. Statistics

Statistical data were obtained using Student's *t*-test and differences with $P < 0.05$ were considered significant.

3. Results

3.1. Butyrate and TSA increased the mRNA II in the rat colon carcinoma cell line CC531

Our previous study showed that the overall GGT activity and the GGT mRNA II levels (using Southern blots) were increased in CC531 cells 48 hr after treatment with butyrate [14]. To establish whether upregulated GGT activity could be a result of increased expression after butyrate and TSA

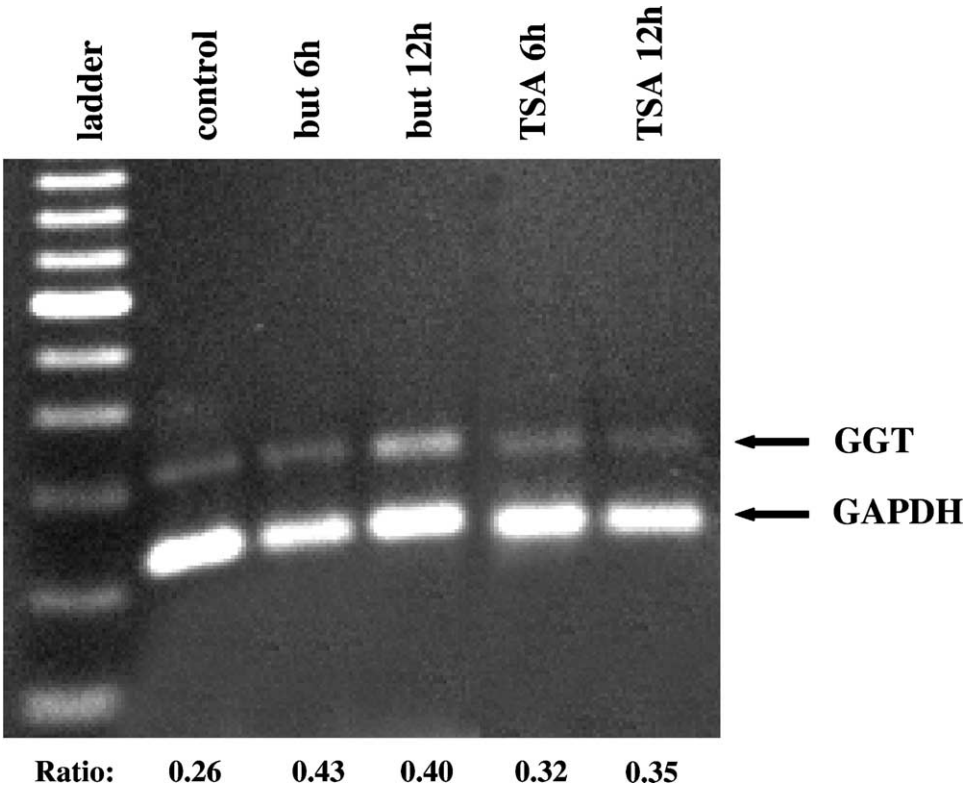

Fig. 2. Stimulation of CC531 cells with butyrate and TSA increases the steady-state levels of transcripts from the GGT P2. Total RNA was extracted from untreated, butyrate-stimulated (2 mM for 6 and 12 hr), or TSA-exposed (0.75 mM for 6 and 12 hr) cells, and subjected to RT–PCR using the primer-dropping method [26]. The amplified fragments were separated on an agarose gel and quantified by scanning after ethidium bromide staining. The ratio numbers indicate the relative density of GGT mRNA II to that of GAPDH.

incubations, we analysed the GGT mRNA II levels in butyrate and TSA exposed cells using quantitative PCR. As shown in Fig. 2, an approximate 150%-fold increase in GGT mRNA II level was detected 6–12 hr after both butyrate and TSA treatments compared to unstimulated cells. Thus, increased GGT activity in butyrate- and TSA-treated cells may result, at least partially, from augmented GGT expression.

3.2. The histone deacetylase inhibitors butyrate and TSA induce the promoter activity of rat GGT P2

Increased GGT mRNA levels can be the result of increased transcription or/and stabilisation of the transcripts. Transient transfection studies using a reporter plasmid with the *luciferase* gene under control of the rat GGT P2 were performed to investigate whether butyrate could exert its effect through increased transcription. A 4-fold enhanced luciferase activity was measured in transfected cells treated with 2 mM sodium butyrate for 24 hr compared to non-treated cells (Fig. 3). Incubations with butyrate for 6 or 12 hr did not result in any increased activity from GGT P2. Incubations for 24 hr with 0.75 μM TSA, another HDAC inhibitor, resulted in a weak (2-fold), but reproducible increase in luciferase activity compared to untreated cells. These data suggest that the effect of butyrate on the rat GGT P2 is not specific, and that other HDAC inhibitors can induce this promoter as well. No increased luciferase activity was observed after butyrate or TSA treatment of cells transfected with the promoter- and enhancer-less reporter plasmid pGL3 (results not shown). This demonstrates that GGT P2 promoter sequences rather than reporter plasmid backbone sequences mediate the effect of butyrate or TSA on luciferase activity.

3.3. Butyrate- and TSA-induced rat GGT P2 activity can be mediated by the Sp1 site

Previous studies have shown that Sp1 binding sites are able to mediate butyrate-induced promoter activity [19,20]. This prompted us to investigate the possible involvement of the GGT P2 Sp1 site in butyrate- and TSA-induction. A triple-copy of the rat GGT P2 Sp1 motif was inserted upstream of a minimal promoter in the luciferase reporter plasmid pTAL-luc. This generated the plasmid pSp1-luc. A 6- to 7-fold induction of the luciferase activity was measured in the pSp1-luc transfected colon carcinoma cells after stimulation with butyrate or TSA (Fig. 4). The level of induction obtained with a triple repeat of the isolated Sp1 motif after butyrate exposure corresponds well with the levels obtained with the whole rat GGT P2. Incubation with TSA, however, yielded a stronger response when the expression of the *luciferase* gene was governed by the three Sp1 motifs linked to the minimal

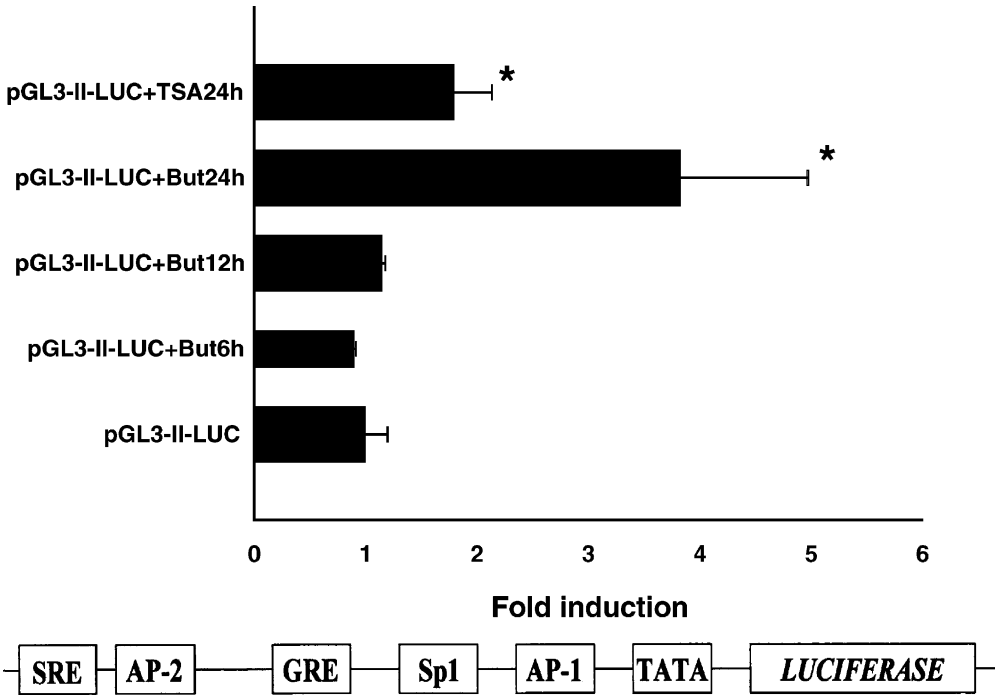

Fig. 3. The histone deacetylase inhibitors butyrate and TSA induce the transcriptional activity of the rat GGT P2. CC531 cells were transiently transfected with the reporter plasmid pGL3-II-luc, containing the rat GGT P2 linked to the *luciferase* gene. Luciferase activity was monitored in untreated cells and in cells stimulated with butyrate (2 mM for 6, 12 and 24 hr) or TSA (0.75 μM for 24 hr). The activity in untreated cells was arbitrary set as 1, while the activity in treated cells is shown as fold induction. The data (±SD) are the means of four independent experiments. An asterisk (∗) indicates a significant difference in luciferase activity ($P < 0.05$) from the untreated, pGL3-II-luc transfected cells. A schematic presentation of the GGT P2 spanning nucleotide −505/+84 is shown in the bottom part of the figure.

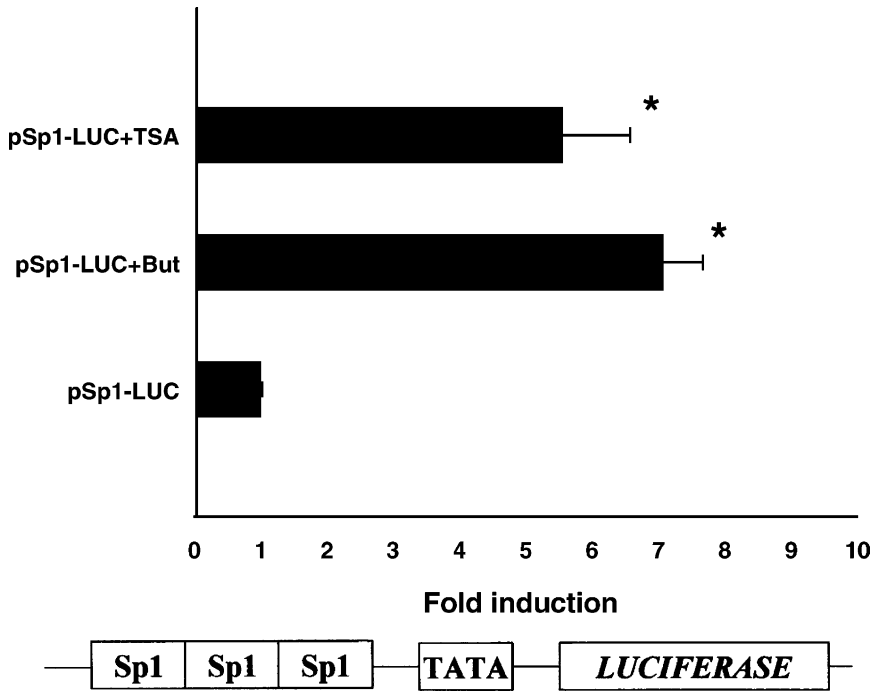

Fig. 4. Isolated Sp1 motifs can mediate butyrate- and TSA-induced activation of the rat GGT P2. Cells were transfected with a luciferase reporter plasmid containing three copies of the putative Sp1 motif present in the rat GGT P2. Stimulations and luciferase activity were as described in the legend of Fig. 3. The data (±SD) represent the means of four experiments. An asterisk (∗) indicates a significant difference in luciferase activity ($P < 0.05$) from the untreated, pGL3-II-luc transfected cells. A schematic presentation of the minimal promoter with three Sp1 motifs is depicted in the bottom part of the figure.

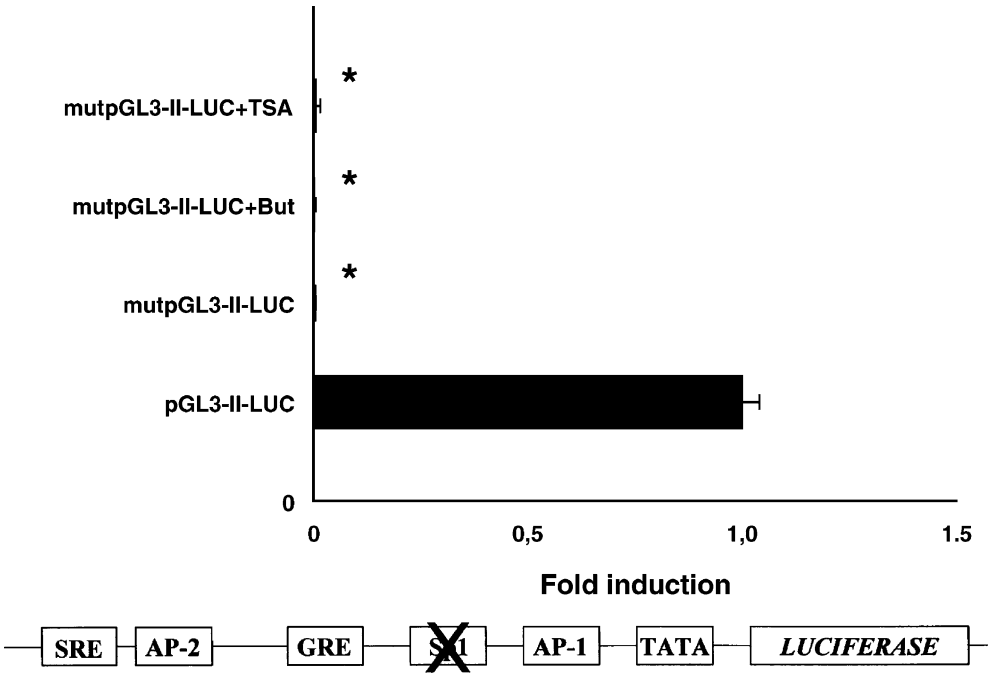

Fig. 5. Mutation in the putative Sp1 site of the rat GGT P2 strongly reduces basal promoter activity and abrogates butyrate- and TSA-induced activation of the mutated promoter. Cells were transfected with the luciferase plasmid pGL3-II-luc, containing the wild type GGT P2 promoter sequences −505/+84, or the plasmid mutpGL3-II-luc in which the putative Sp1 site in the GGT P2 sequences was mutated. Stimulations and luciferase activity were as described in the legend of Fig. 3. The data (±SD) represent the means of four experiments. An asterisk (∗) indicates a significant difference in luciferase activity ($P < 0.05$) from the untreated, pGL3-II-luc transfected cells. A schematic presentation of the Sp1-mutated GGT P2 spanning nucleotide −505/+84 is drawn in the bottom part of the figure. The actual mutation in the Sp1 motif is given in Fig. 1.

promoter in the pTAL-luc plasmid as compared to the expression of the −505/+84 GGT P2 fragment (Fig. 3). These results proof that the Sp1 binding is, at least partially, responsible for butyrate- and TSA-induced activation of the rat GGT P2. However, other motifs may also contribute to mediate the effect of these HDAC inhibitors on the activity of the rat GGT P2.

3.4. Mutating the Sp1 site in the context of rat GGT P2 strongly reduced basal activity and abrogated butyrate- and TSA-induced activation of the mutated promoter

Next, the unique Sp1 site was destroyed by site-directed mutagenesis in the −505/+84 fragment of rat GGT P2 promoter. Transient transfection studies with this construct almost completely abolished the basal activity of this promoter and abrogated the butyrate-induced activation (Fig. 5). Similarly, TSA was unable to activate this mutated promoter. These observations emphasise the importance of the Sp1-binding site in basal activity of this promoter and strengthen the findings that the Sp1 motif is important in mediating butyrate- or TSA-induced activation of rat GGT P2.

4. Discussion

Butyrate is a well-known inducer of differentiation in various cell types and is a potent inhibitor of HDACs [27,28]. Treatment of the rat colon carcinoma cell line CC531 with butyrate and TSA resulted in differentiation, induction of apoptosis and also increased GGT activity [14]. Using semi-quantitative RT–PCR we found elevated GGT mRNA II, 6–12 hr after both butyrate and TSA treatments. The present study was directed at defining the mechanism(s) involved in butyrate-induced GGT activity. Our experiments here demonstrated that butyrate, as well as another HDAC inhibitor, TSA, could evoke increased GGT activity through Sp1-dependent activation of GGT P2 resulting in enhanced levels of GGT mRNA II.

The role of Sp1 in butyrate- and TSA-induced activation of the GGT P2 promoter was examined by transient transfection studies and not on the promoter in its natural chromatin context. Evidence exists that promoter sequences in transient transfected plasmid DNA can be packed in a chromatin-like structure. A previous study has shown that plasmid DNA rapidly assembled into mini-chromosomes containing nucleosomes with a typical 190 bp repetitive spacing. Treatment of transfected cell with Na-butyrate increased the DNase I sensitivity of the plasmid DNA. These results suggested that transfected plasmid DNA is converted into a chromatin-like conformation that can be modified by HDAC inhibitors [29]. Another group found that transiently transfected plasmid DNA became rapidly packed with histones, but micrococcal nuclease digestion indicated the assembly of atypical chromatin structures [30]. The fact that GGT P2 transcripts increased in butyrate- and TSA-treated cells

correlates well with the transient transfection studies that demonstrated increased GGT P2 promoter activity after exposure to these HDAC inhibitors. We cannot, however, exclude that the effect of butyrate or TSA on endogenous GGT P2 mRNA levels was partially the result of stabilisation of these agents. So far, no prevalence for butyrate or TSA-induced stabilisation of mRNA has been reported to our knowledge. In fact, butyrate has been demonstrated to destabilise some transcripts [31,32].

It has become obvious from recent research that nucleosome remodelling through histone deacetylases and histone acetyl transferases (HATs) plays an important role in transcriptional regulation [33]. Butyrate and TSA are often used to study the involvement of chromatin remodelling on the transcriptional activity of promoters. Both compounds have been shown to induce transcription through Sp1 binding sites in susceptible promoters. Induction of expression by sodium butyrate or TSA of the murine *thymidine kinase* gene, the human *dihydrofolate reductase* gene, the *alkaline phosphatase* gene, the $p21^{WAF1/Cip1}$ promoter, the mouse *galectin-1* gene, the human adenine nucleotide translocase isoform 2 promoter, the chicken *stearoyl-coA desaturase 1* gene, the human $G_{i\alpha}$ gene, and the human immunodeficiency virus type I long terminal repeat all depended on the integrity of Sp1 sites [19,34,35]. Moreover, Hodny *et al.* [36] claim that HDAC inhibitors are not able to activate a promoter that lacks Sp1 sites.

The mechanisms by which HDAC inhibitors exert their effect on Sp1 begin to emerge. Several groups have shown that the histone deacetylase-1 (HDAC-1) can associate with Sp1 *in vivo* [34,37]. Butyrate or TSA may thus relieve the inhibitory effect of Sp1-bound HDAC-1, resulting in increased transcriptional activity of the promoter [37]. Sp1 may also recruit chromatin remodelling factors with HAT activity which can antagonise the inhibitory effect of HDAC-1. Co-expression of p300, a co-activator with intrinsic HAT activity, stimulated the $p21^{WAF1/Cip1}$ promoter through both Sp1 and Sp3 binding sites, while a dominant-negative p300 mutant blocked TSA-induced $p21^{WAF1/Cip1}$ promoter activation [38]. p300 was shown to interact directly with Sp1 and to stimulate GAL4-Sp1-mediated transcription in HeLa cell extracts [39]. Recently, it was demonstrated that Sp1 stimulated the HAT activity of the CREB-binding protein CBP, a general co-activator related to p300 [40]. These findings exemplify that HDACs, e.g. (HDAC-1) and HATs (e.g. p300, CBP) can collaborate with Sp1 to repress or activate transcription of certain genes. In addition to recruitment of proteins with HDAC or HAT by Sp1, binding of Sp1 to its cognate motif is highly affected by the structure of the nucleosomes. The affinity of the Sp1 protein for its binding site is diminished upon the formation of nucleosomes on naked DNA *in vitro* [41]. The chromatin-structure forms therefore a major determinant for repression or activation of gene expression as it is dependent on the interaction between several ''nucleosome-remodelling'' activities [42,43]. This regulatory mechanism has been described in the adenine–nucleotide–translocase-2 promoter (ANT-2). The Sp1 sites in the proximal promoter of ANT-2 in the chromatin were found to be involved in the repression of this promoter in unstimulated cells. *In vitro* studies of this promoter indicated that both of the Sp1 sites in the proximal promoter of ANT-2 were needed for an increased transcription in the transiently transfected cells exposed to butyrate and TSA. The authors speculated that reminiscent ANT-2 promoter activity in untreated cells was caused by hypoacetylated nucleosomes that were able to suppress the promoter activity in the absence of the HDAC inhibitors.

Sp1-independent mechanisms for butyrate- or TSA-induced promoter activity exist. Indeed, not all promoters with Sp1 sites are activated by the HDAC inhibitors [33]. Moreover, disruption of Sp1 sites did not interfere with butyrate-induced activity of the integrin CD11c promoter [44,45]

In conclusion, our results clearly demonstrate a role for the Sp1 site in activation of the rat GGT P2 promoter by the HDAC inhibitors butyrate and TSA. This suggests that butyrate and TSA relieve the inhibitory effect of HDAC which can associate with Sp1 *in vitro*. Recruitment of proteins with HAT activity by Sp1 may be an additional mechanism involved in the regulation of the GGT P2 promoter. The contribution of other transcription factors binding sites was not investigated, but mutating the Sp1 site strongly impaired the basal activity of the GGT P2 and abolished the induction of this promoter by the HDAC inhibitors. Mutations of individual putative transcription factor binding sites should enable us to establish the contribution of other regulatory elements during butyrate-mediated GGT P2 activation.

Acknowledgments

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