Analysis of *c-fos* expression in the butyrate-induced F-98 glioma cell differentiation

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The functional induction of *c-fos* in the sodium butyrate-induced differentiation of F-98 glioma cells was studied. Fos protein level was increased by butyrate. In contrast, c-Jun protein was constitutively expressed and was not affected by butyrate. Gel-retardation assay indicates Fos as a component of the complex formed between the consensus oligonucleotide of the TPA (PMA, phorbol 12-myristate 13-acetate) response element (TRE) and nuclear extract prepared from butyrate-treated cells. Transfection studies showed that butyrate increased transcription from a multimeric TRE-driven reporter construct, and the effect was mimicked by transfecting cells with *fos*-expression plasmid. Furthermore, under conditions of *c-fos* over-expression, transactivation by butyrate was essentially abolished. These data suggest that Fos induction had a functional role in gene activation. Characterization of stable *c-fos* transfectants demonstrated that these cells displayed alterations in morphology,

showed serum-dependent growth, had slower growth rates and grew to lower saturation densities than did untransfected F-98 cells or transfected cells that did not express *c-fos*. Immunofluorescent staining indicated that *fos* transfectants also had elevated glial fibrillary acidic protein ('GFAP') expression. Transfection of the *c-fos* promoter–chloramphenicol acetyltransferase fusion gene into F-98 cells revealed that activation of *c-fos* by butyrate was exerted at the promoter level, and sequences located within nucleotides –757 to –402 of the *c-fos* promoter were responsible for butyrate induction. Our data indicate that transcriptional activation of *c-fos* through its promoter by butyrate resulted in increased Fos protein expression. Transfection studies show that both *c-fos* and butyrate activate TRE-containing genes, and *fos* may be a downstream mediator of butyrate. Furthermore, expression of *c-fos* plays a major role in modulating the growth properties of F-98 cells.

INTRODUCTION

Proto-oncogenes play important roles in cell growth and differentiation. This is suggested by the finding that oncogene products are growth factors or growth-factor receptors (for reviews, see [1,2]). Moreover, induction of cellular growth or differentiation in a wide variety of cells is often accompanied by simultaneous changes in oncogene levels. For example, an initial down-regulation of the *c-myc* gene was often found in erythroleukaemia cells induced to differentiation by a number of agents [3,4]. Transfection of the sense or antisense *c-myc* gene further suggested that a prior decrease in *c-myc* expression was a prerequisite for erythroleukaemia-cell differentiation [5]. The finding that expression of a dominant inhibitory *Ha-ras* mutant in PC12 pheochromocytoma cells inhibited the differentiation induced by NGF [6] also indicates a role of *c-ras* in differentiation.

The *c-fos* gene was also found to be inducible in numerous cell systems by a wide variety of stimuli including serum, phorbol ester [7], epidermal growth factor [8], NGF [9], and oncogenes such as *src* [10], *ras* [11] etc. It seems that *c-fos* expression correlates with cell proliferation. However, in cells induced to differentiate, elevated amounts of *c-fos* transcript have also been noted. Examples include the differentiation of PC12 cells induced by NGF [12], *src* or *ras* [13], and in HL-60 and U-937 monomyelocyte differentiation induced by PMA [14,15]. Although a preponderance of evidence shows elevated *c-fos* gene expression in differentiating cells, much more needs to be done to define the precise role of the induced *c-fos* transcripts in the process.

Studies on the transcriptional regulation of the *c-fos* gene have revealed the presence of multiple cis-acting elements. These include an element essential for transcriptional activation by

serum [16], two cyclic-AMP response elements [17], a *sis*-inducible element [18,19], an Apl-like element [20], and multiple basal promoter elements [21]. In addition to the regulation by 5'-enhancer sequences, the steady-state levels of *c-fos* mRNA are also regulated post-transcriptionally [22,23], and the 3'-untranslated region containing AU-rich sequence was reported to be responsible for the rapid turnover of the *c-fos* mRNA [24,25]. Fos protein is a nuclear protein that belongs to the leucine-zipper family of transcription factors; it contains a basic region immediately adjacent to the leucine repeat [26,27]. This structure is common to a number of transcription factors such as cAMP response element binding protein (CREB), Myc, and Jun. It has been shown that *c-fos* protein forms a heterodimer with Jun and regulates the expression of PMA-response genes by binding to TRE [28–30]. It is therefore not surprising to find that the activation of *c-fos* is an important nuclear signalling mechanism used by many molecules, since expression of this trans-regulator will result in a cascade of secondary-response gene expression which may regulate a specific programme of growth or differentiation.

Sodium butyrate exhibits a wide variety of complex biological effects. It alters gene expression, and it inhibits the growth and induces the differentiation of many cell types [31]. We have previously reported that *c-fos* gene was rapidly induced following exposure of F-98 rat glioma cells to butyrate [32]. The mechanism of *c-fos* activation and the function of its expression during the differentiation, however, remain unexplained. In the present study, we have shown that the 5'-regulatory region of *c-fos* gene is responsive to butyrate activation. In addition, we demonstrated that *c-fos* expression did result in the activation of a DNA construct composed of a promoter containing TRE sequence

Abbreviations used: CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GFAP, glial fibrillary acidic protein; NGF, nerve growth factor; PMA, phorbol 12-myristate 13-acetate ('TPA'); TRE, TPA-response element.

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coupled to the enzyme CAT. Furthermore, analysis of cell clones that expressed *c-fos* constitutively indicates a critical role of *c-fos* in regulating the growth properties of F-98 cells.

MATERIALS AND METHODS

Materials

DMEM, fetal-calf serum, glutamine, geneticin, RNAase T1, S1 nuclease and Proteinase K were purchased from GIBCO BRL. T7 RNA polymerase, RQ 1 DNAase I, pGEM3Zf+, pCAT/promoter, *in vitro* transcription kit and AP1 consensus (TRE) oligonucleotide used in gel-retardation analysis were from Promega Corp. Plasmids *pc-fos-1* (human), *pc-fos-3* (mouse), pRSV2cat and pSV2neo were from American Type Culture Collection. Restriction and modifying enzymes were purchased from Boehringer Mannheim. Sodium butyrate was obtained from Baker Chemical Co. Rabbit polyclonal antibody against Fos was from Santa Cruz Biotechnology, and anti-Jun antibody was from Oncogene Science. Monoclonal antibody to glial fibrillary acidic protein (GFAP) was from DAKO Company. Radioisotopes were obtained from Amersham Corp. PMA, RNAase A, Protein A-agarose, penicillin and streptomycin were purchased from Sigma.

Cell culture

F-98 glioma cells were maintained in DMEM supplemented with 10 % fetal-calf serum, 4 mM glutamine, penicillin (200 i.u./ml) and streptomycin (200 µg/ml). The cultures were incubated in a humidified incubator with 10 % CO₂ at 37 °C; cells were sub-cultured every 3 days. For the butyrate treatment, 8×10^5 cells were seeded in a 10 cm-diameter tissue-culture dish for 48 h, and then exposed to 2.5 mM sodium butyrate for various intervals of time as stated.

Plasmid DNA

p5xTRE/TK-CAT containing five copies of the TRE sequence (ATGAGTCA) of the human collagenase gene and pΔ72/TK-CAT containing mutated TRE sequence (GAGTCA) on the gene were obtained from Dr. Michael Karin of the University of California at San Diego. Schematic representation of the plasmids used in the present study is shown in Figure 1. pRSV/*Hind*III was derived from pRSV2cat by digesting pRSV2cat with *Nru*I followed by *Hind*III linker ligation. The plasmid was cleaved with *Hind*III to remove excess linker and re-ligated. pRSV/*fos*/*Nae*I was constructed by insertion of the *c-fos* coding fragment derived from *pc-fos-3* containing mouse genomic DNA to pRSV/*Hind*III. Briefly, *pc-fos-3* was digested with *Nae*I to generate a segment of *c-fos* DNA composed of sequences from +175 to +2727. The fragment was ligated to *Hind*III linker and cloned into the *Hind*III sites of pRSV/*Hind*III. The orientation of the inserted gene was verified by digesting with *Eco*RI. Plasmid (pGEM/*fos*/*NHc*) for synthesizing *fos* probe used in the RNA protection analysis was constructed as follows: pGEM/*fos*/*NHc* containing a fragment from +175 to +415 of mouse *c-fos* genomic DNA was obtained by digesting *pc-fos-3* with *Nae*I (+175) and *Hinc*II (+415), followed by ligation with *Hind*III linker. The 241 bp fragment was then subcloned into the *Hind*III site of the pGEM3Zf+. The orientation was identified by DNA sequencing.

Plasmids pF750, pFC2 and pFC9 (Figure 1b) were constructed as described by Deschamps et al. [33], except that vector pGEM3Zf+ was used. pFCBS was obtained by digesting pF750 with *Sst*II (−402) and *Apa*I (−225); the sites were made blunt with Klenow enzyme and re-circularized by blunt-end ligation.

Nuclear-extract preparation

Cells (2×10^7) were collected in PBS and pelleted at 500 *g* for 5 min at 4 °C. The cells were first resuspended in 1 ml of lysis buffer (10 mM Tris/HCl, 140 mM KCl, 5 mM MgCl₂, 1 mM DTT, pH 7.9) containing freshly added protease inhibitors (0.25 mM phenylmethanesulphonyl fluoride and 2.5 µg each of antipain, leupeptin, chymostatin and pepstatin A). Then 1 ml of lysis buffer containing 1 % Nonidet P-40 was then added. The cells were incubated for 5 min on ice and nuclei collected by centrifuging at 500 *g* for 5 min at 4 °C. The nuclei were washed with 1 ml of cold wash buffer (20 mM Tris/HCl, 20 % glycerol, 140 mM KCl, 10 mM MgCl₂, 1 mM DTT) and resuspended in 500 µl of nuclear extraction buffer (0.35 M NaCl, 5 mM EDTA, 1 mM DTT, 10 mM Hepes, pH 7.5) containing the above-mentioned protease inhibitors. After 30 min at 4 °C with periodic stirring, the mixture was centrifuged at 10000 *g* for 15 min. Supernatant containing the nuclear extract was used immediately or stored at −70 °C in the presence of 10–20 % glycerol.

Gel-mobility-shift assay

Nuclear extract (10 µg) was mixed in 25 µl of gel-shift buffer (10 mM Hepes, 50 mM NaCl, 0.1 % Nonidet P-40, 10 % glycerol, 1 mM EDTA, 1 mM DTT, 5 mM MgCl₂, pH 7.5) containing 2 µg of poly(dI-dC). The mixture was incubated at room temperature for 30 min in the presence or absence of anti-Fos antibody or normal rabbit immunoglobulin. After adding 0.1 pmol of the labelled TRE oligonucleotide and incubating for an additional 30 min, the DNA–protein complex was resolved from free nucleotide by electrophoresis on a 4 % polyacrylamide gel (acrylamide:bisacrylamide 19:1, w/w) in Tris/borate buffer (22.5 mM Tris/borate, 0.5 mM EDTA, pH 8.0). After transfer to nitrocellulose paper, the binding was detected by exposing the paper to an X-ray film.

Western blotting

F-98 cells (1×10^6 cells) were plated on a 10 cm tissue-culture dish for 48 h. Cells were collected and washed with PBS. Total cell lysates were obtained by mixing the cells with 5 ml of lysis buffer (0.5 % deoxycholate, 1 % Triton X-100, 0.1 % SDS, 1 mM NaF, 0.25 mM phenylmethanesulphonyl fluoride, 10 mM phosphate buffer, pH 7.2), followed by repeated aspiration through a 21-gauge needle. Cell lysates were transferred to a centrifuge tube, and the cell debris was removed by centrifuging at 10000 *g* for 45 min at 4 °C. After SDS/PAGE in an 8 % polyacrylamide gel, the proteins were transferred to a nitrocellulose paper. The paper was soaked in 5 % non-fat milk for 45 min to decrease non-specific binding, and then made to react with specific antibodies dissolved in 5 % non-fat milk for 45 min. Membrane was washed for 2 × 7 min with 10 mM Tris/HCl/150 mM NaCl, pH 8.0 (TBS), containing 0.05 % Tween 20, followed by incubating with horseradish-peroxidase-conjugated goat anti-rabbit (for Fos and Jun) antibody at 1:3000 dilution in 5 % non-fat milk for 30 min. The membrane was washed for 3 × 5 min with TBS containing 0.05 % Tween 20, and once for 5 min with TBS. Signals were detected by treating with ECL reagents (Amersham International) for 1 min, followed by exposure to an X-ray film.

Transcription *in vitro* and RNAase protection analysis

Total cellular RNA from cultured cells was isolated by disrupting the cells with guanidine isothiocyanate followed by CsCl centrifugation as described by Chirgwin et al. [34].

Figure 1 Diagrammatic representation of plasmids constructed in the study

(a) Plasmids used in the *c-fos* transfection studies. $\square$ indicates the promoter regions and $\blacksquare$ denotes the coding sequence or exons. Construction details are described in the Materials and methods section. (b) Plasmids of the *c-fos* promoter-CAT gene constructs used in the analysis of sequence responsive to butyrate induction. As described in the Materials and methods section, the constructs contain parts of the human *c-fos* promoter, extending from variable 5' sites to position +42, fused to the CAT reporter gene. The transcriptional initiation site is indicated by an arrow.

A 1 μ g portion of the plasmid DNA (pGEMfos/NHc) was linearized with *Pvu*II. Transcription *in vitro* was allowed to proceed by adding T7 RNA polymerase (reaction temperature 42 °C) to a mixture containing linearized DNA, nucleotides and [³²P]UTP in transcription buffer for 60 min [35]. After the reaction, DNA was removed with RQ1 RNAase-free DNAase I and the unincorporated nucleotides were removed by ammonium acetate precipitation. The RNA probe was dissolved in 400 μ l of TE buffer (10 mM Tris/HCl/1 mM EDTA, pH 7.4) and stored at -20 °C.

A 20 μ g sample of RNA was mixed with (1–5) $\times 10^5$ c.p.m. of the RNA probe in 30 μ l of hybridization buffer (40 mM Pipes, 1 mM EDTA, 0.4 M NaCl, 80 % formamide, pH 6.4). The mixture was denatured at 85 °C for 10 min, and hybridization was carried out at 47.5 °C for 8–12 h. At the end of the incubation, 300 μ l of RNAase digestion mixture (300 mM NaCl, 10 mM Tris/HCl, pH 7.4 containing 5 mM EDTA, 2 μ g/ml RNAase T1

and 40 μ g/ml RNAase A) was added and the digestion was performed at 37 °C for 60 min. The mixture was treated with proteinase K at 37 °C for 30 min, followed by phenol/chloroform extraction and ethanol precipitation. RNAase-resistant fragment was separated on a 6 % polyacrylamide/6 M urea gel and the radioactivity was detected by autoradiography.

Transfection

F-98 cells (8×10^5) were plated on a 10 cm tissue-culture dish 24 h before transfection by the calcium phosphate method; 4 h before transfection, the medium was changed. DNA to be transfected was adjusted to a final amount of 20 μ g with pGEM3Zf+ DNA. The cells were exposed to the DNA-calcium phosphate precipitate for 12–16 h. Cells were washed and incubated with fresh medium for another 12–18 h. They were then treated with 2.5 mM sodium butyrate for 18 h; lysates were prepared and analysed for CAT activity.

Stable transfectants were isolated as follows: F-98 cells were co-transfected with 20 μg of pRSV*fos*/*NaeI* and 1 μg of pSV2neo. At 48 h after transfection, cells were diluted in a ratio of 1:10 and grown in the continuous presence of G418 (1 mg/ml). The cells were cultured in the presence of G418, with medium changed every 3 days. Stable G418-resistant clones were isolated after 18 days.

CAT assay

Cell extracts were prepared by three cycles of freezing at -70°C and thawing at 37°C . The extracts were heated at 65°C for 10 min to remove the intrinsic deacetylase activity present in the cells. CAT activity was determined by incubating the extract with acetyl-CoA and [^{14}C]chloramphenicol as described by Gorman et al. [36]. Acetylated products were separated by t.l.c. and detected by autoradiography. Spots on the thin layer containing the radioactivity were scraped off and the radioactivities determined.

Immunofluorescence staining

Cells ($5 \times 10^4/200 \mu\text{l}$) were seeded on to coverslips and cultured for 24 h. After excess serum was washed off, the cells were fixed with acetone for 5 min at 4°C and dried in air. Cells were rehydrated by incubating with PBS for 10 min, and blocked with 3% non-fat milk for 30 min. After incubation with monoclonal antibody to GFAP overnight at 4°C , cells were washed extensively with PBS (8×5 min), blocked with 5% non-fat milk, and incubated with fluorescein isothiocyanate-labelled goat anti-mouse antibody. Cells were examined under a fluorescence microscope (Leitz Laborlux K) at a magnification of $\times 400$.

RESULTS

Butyrate increases Fos protein expression, but does not affect Jun levels

Previous studies indicate that *c-fos* gene was induced 30 min after

Figure 2 Western-blot analysis of Fos and Jun protein expression

F-98 cells were treated with sodium butyrate (2.5 mM) for 2 h; cells were then lysed and nuclear extract was prepared as described in the Materials and methods section. The proteins (15 μg) were separated by SDS/PAGE (8% gel), transferred to a nitrocellulose paper, and incubated with specific antibodies to c-Fos (**a**) or c-Jun (**b**). After washing, the blot was incubated with horseradish-peroxidase-conjugated goat anti-rabbit antibody, followed by reaction with enhanced chemiluminescence reagents (Amersham International) and exposure to an X-ray film as described in the Materials and methods section. Molecular masses of prestained marker proteins are shown in kDa. Lanes are, in (**a**): 1, control F-98 cells; 2, cells treated with sodium butyrate for 2 h; 3, F-98 cells stably transfected with *c-fos* (clone 1-15); in (**b**): 1, control F-98 cells; 2, cells treated with sodium butyrate for 2 h. The positions of Fos and Jun are indicated by arrows.

cells were exposed to butyrate [32]. Results from this laboratory (not shown) demonstrate that *c-jun* mRNA was constitutively expressed in F-98 cells, and its level was not affected by butyrate treatment. Fos itself does not bind DNA; instead, it forms a heterodimer with Jun, and the Fos/Jun complex, in turn, activates transcription of the genes containing TRE. Therefore, we examined the expression of both Fos and Jun protein levels in F-98 cells by Western-blot analysis. Nuclear extracts were prepared from control and butyrate-treated cells and separated by SDS/PAGE. As depicted in Figure 2(a), anti-Fos antibody specifically recognized a protein with a molecular mass equivalent to that of Fos. This protein was not detected in the untreated cells (lane 1) or in immunoblotting with normal rabbit serum (results not shown). Control experiments performed with F-98 cells stably transfected with *c-fos* DNA (clone 1-15, see below) also showed the presence of this protein (lane 3). These results indicate that Fos protein was induced by butyrate. On the other hand, immunoblotting with anti-Jun antibody showed that F-98 cells constitutively expressed the 39 kDa Jun protein, and its level was not changed by butyrate (Figure 2b). The expression of Fos and Jun proteins paralleled their mRNA expression. It appeared that, although Fos and Jun co-ordinate tightly in their function to activate responsive genes, their expression is under independent regulation in cells.

Butyrate and *c-fos* transfection both stimulate the TRE-dependent transcriptional activity

Since *c-jun* is constitutively expressed in F-98 cells, it is likely that, after butyrate treatment, elevated *c-fos* expression caused an increase in Fos/Jun dimer formation. This, in turn, bound to and activated promoters containing the TRE sequence. To investigate whether in the butyrate-treated cells Fos was able to bind to the TRE oligonucleotide, we carried out DNA-binding experiments. The gel-mobility shift assay (Figure 3) indicates that

Figure 3 Gel-mobility-shift assay of nuclear extracts from butyrate-stimulated cells

Nuclear extracts were prepared as described in the Materials and methods section. For DNA-binding experiments, 15 μg of extracts from untreated, butyrate-treated, or stably *fes*-transfected (clone 1-15) F-98 cells were mixed with 0.1 pmol of ^{32}P -labelled TRE oligonucleotide, and the protein-DNA complex was separated from free DNA by gel electrophoresis on a 4% polyacrylamide gel. Binding was also performed in the presence of a 10-fold molar excess of unlabelled TRE oligonucleotide ('10 × competitor DNA'), or in the presence of normal rabbit serum ('Pre-immune serum') or polyclonal antibodies to c-Fos as indicated. Lanes: 1, untreated F-98 cells; 2, F-98 cells treated with butyrate for 2 h; 3, stably *fes*-transfected clone.

Table 1 Stimulation of the trans-activating activity from the TRE-containing CAT reporter construct by butyrate

F-98 cells were transiently transfected with 5 μ g of p5xTRE/TK-CAT (5xTRE) or p Δ 72/TK-CAT (Δ 72) plus 15 μ g of pGEM3Zf+; 12–16 h later, medium was changed and the incubation was continued for 18 h. Cells were then treated with (+) or without (–) 2.5 mM sodium butyrate for 18 h. At the end of the incubation, cell extracts were prepared; 10 μ g samples of lysate proteins were analysed for CAT activity. CAT activity values are expressed as pmol of acetylated chloramphenicol/h per mg of cell-lysate protein, or relative to the activity of the 5xTRE-transfected cells in the absence of butyrate exposure, and represent means ($\pm$ S.D.) from six independent transfections. Fold induction is defined as the CAT activity measured in the presence versus that in the absence of sodium butyrate treatment. Note that the large divergence in the absolute CAT activity is due to the variation of transfection efficiency between different experiments. The relative activity and fold induction are, however, independent of transfection efficiency, as indicated by the smaller deviation.

Reporter gene	CAT activity (pmol/h per mg)		Relative activity		Fold induction
	–	+	–	+	
5xTRE	4711 $\pm$ 1025	16211 $\pm$ 6673*	1	3.02 $\pm$ 0.33	3.02 $\pm$ 0.33
Δ 72	879 $\pm$ 250	1104 $\pm$ 227†	0.19 $\pm$ 0.05	0.21 $\pm$ 0.05	1.10 $\pm$ 0.12

* Significant difference ($P < 0.001$) by comparison with the CAT activities of 5xTRE-transfected cells in the absence of butyrate exposure (by Student's t test).

† No significant difference ($P > 0.1$) by comparison of the CAT activities with those in the absence of butyrate exposure in Δ 72-transfected cells.

nuclear extract from the untreated F-98 cells contained proteins that bound to the TRE sequence. This is not unexpected, since c-Jun constitutively expressed in F-98 cells (Figure 2) is able to form a homodimer and bind to TRE oligonucleotide. In cells treated with butyrate for 2 h, a moderate but consistent increase in binding was observed (1.9-fold, $P < 0.02$, from four independent experiments). The specificity of the binding was evidenced by the observation that no significant binding could be detected in the presence of excess of unlabelled TRE oligonucleotide. To determine if the protein complex bound to the TRE oligonucleotide contained c-Fos, mobility-shift analysis was performed with nuclear extracts preincubated with antibodies to Fos. As shown in Figure 3, antibodies to c-Fos supershifted a portion of the protein–DNA complex in extracts from the butyrate-treated cells, but not that from the untreated cells. Control experiments performed with a *c-fos* transfectant (lane 3) showed a greater extent of binding; anti-Fos antibodies were able to retard the protein–DNA complex formed. These results suggest that Fos is participating in specific recognition of the TRE oligonucleotide in the butyrate-stimulated cells.

By transiently transfecting F-98 cells with a DNA construct containing five copies of TRE coupled to the CAT reporter gene (p5xTRE/TK-CAT), we found that butyrate treatment resulted in a significant increase ($P < 0.001$ by Student's t test) in CAT activity (Table 1). Although the absolute CAT activity varied between each transfection, the fold induction by butyrate remained relatively constant. Results from six independent experiments gave an average of approx. 3-fold increase in CAT activity in the butyrate-treated cells. Controlled experiments with CAT gene coupled to a mutated TRE (p Δ 72/TK-CAT) did not show any activation in CAT by butyrate. These results suggest that transcriptional regulation accounts, at least in part, for the activation of TRE-containing gene expression by butyrate.

We next tested the possibility that *c-fos* expression in F-98 cells can indeed activate the TRE-dependent gene. Mouse *c-fos*-expression plasmid driven by the RSV promoter (pRSV*fos*/NaeI) was co-transfected with p5xTRE/TK-CAT (5xTRE). Results (Table 2) indicate that transfection of *c-fos* gene (*fos*), when compared with the vector control using pRSV/*Hind*III (RSV), did activate CAT activity ($P < 0.001$ by Student's t test). CAT activity in control experiments utilizing mutated TRE (Δ 72) was not affected by *c-fos* gene transfection. Furthermore, in the presence of *c-fos* expression, the ability of butyrate to stimulate the activity of CAT driven by TRE was essentially abolished

Table 2 Trans-activation of TRE–CAT reporter construct by the transfected *c-fos*

F-98 cells were co-transfected with 5 μ g of p5xTRE/TK-CAT (5xTRE) or p Δ 72/TK-CAT (Δ 72) plus 15 μ g of *fos*-expression plasmid pRSV*fos*/NaeI (*fos*) or vector control pRSV/*Hind*III (RSV) as indicated. Experimental procedures and other details are described in Table 1. CAT activity values are expressed as pmol of acetylated chloramphenicol/h per mg of cell-lysate protein, or relative to the activity of the 5xTRE- and vector-control (RSV)-transfected cells, and represent means ($\pm$ S.D.) from four independent transfections.

Reporter gene	Expression vector	CAT activity (pmol/h per mg)	Relative activity
5xTRE	RSV	6127 $\pm$ 912	1
5xTRE (– butyrate)	<i>fos</i>	25839 $\pm$ 5212†	3.88 $\pm$ 0.55
5xTRE (+ butyrate)*	<i>fos</i>	31732 $\pm$ 8764‡	(1.32 $\pm$ 0.12)
Δ 72	RSV	656 $\pm$ 403	0.12 $\pm$ 0.06
Δ 72	<i>fos</i>	761 $\pm$ 293§	0.18 $\pm$ 0.07

* To obtain the butyrate stimulation in the presence of *fos* expression, cells were treated with 2.5 mM butyrate after co-transfection as described in Table 1. The number in parentheses was derived by dividing CAT activity in the presence by that in the absence of butyrate treatment.

† Significant difference ($P < 0.001$) by comparison of the CAT activities with those obtained from the 5xTRE and vector control (RSV) co-transfection (by Student's t test).

‡ No significant difference ($P > 0.2$) by comparison with the CAT activities from the 5xTRE- and *fos*-co-transfected cells in the absence of butyrate exposure.

§ No significant difference ($P > 0.1$) by comparison with the CAT activities from the Δ 72- and RSV-co-transfected cells.

(compare CAT activity in *c-fos*-co-transfected cells in the presence or absence of butyrate).

To verify that *c-fos* was indeed expressed in transiently transfected cells, RNAase-protection analysis was employed utilizing the 241-nucleotide fragment (+175 to +415) of the mouse *c-fos* gene as a probe. For this particular experiment, RNAase digestion was performed at 37 °C; at this temperature, only the transfected mouse transcripts could be detected because of the presence of several sequence mismatches in the protected region between the rat and mouse *c-fos* genes. As shown in Figure 4, the vector-transfected control (RSV) confirms that the condition chosen was specific for detecting mouse transcript only, since butyrate activation of the endogenous *c-fos* expression was not discernible in these cells. On the other hand, expression

Figure 4 RNAase-protection analysis of *c-fos* RNA expression in the transfected cells

The upper diagram represents the structure of the pRSVfos/NaeI transcription unit, which contains the mouse *c-fos* genomic DNA fused to the RSV promoter. The T7 RNA probe was generated from pGEMfos/NHc as described in the Materials and methods section (abbreviation: nt, nucleotides). The lower diagram indicates the results from RNAase-protection assays. F-98 cells were transfected with pRSV/HindIII (RSV) or pRSVfos/NaeI (fos); the cells were then treated with (B) or without (C) butyrate as described in Table 2. A 20 μ g sample of RNA was hybridized with radiolabelled T7 RNA probe, and the mixture was then digested with RNAase at 37 $^{\circ}$ C. Size markers (M; numbers of nucleotides) are shown at the left. The protected fragment that corresponds to *c-fos* RNA is indicated.

Table 3 Formation of colonies after transfecting F-98 cells with *c-fos*

F-98 cells (1×10^6 cells) were plated in a 100 mm culture dish; cells were co-transfected with 20 μ g of pRSV/HindIII (RSV) or pRSVfos/NaeI (fos) plus 1 μ g of pSV2neo. The number of G418-resistant (Neo^r) colonies were scored after 18 days. Numbers are from two independent experiments, and are expressed as means $\pm$ S.D.

Plasmids	No. of Neo ^r colonies/plate		
	Total	Large colonies (diameter > 3 mm)	Small colonies (diameter < 3 mm)
RSV	246 $\pm$ 10	153 $\pm$ 14	93 $\pm$ 7
fos	107 $\pm$ 12	56 $\pm$ 10	51 $\pm$ 9

of exogenous mouse *c-fos* mRNA was found in the *c-fos*-transfected cells. The observation that butyrate also activates the RSV promoter is evidenced by increased *c-fos* transcript levels in the butyrate-treated cells. This supports the previous studies that butyrate increased RSV promoter activity [37]. Besides the 241-nucleotide sequence expected to be protected, two more species of lower mobility were detected. One possible explanation is the presence of a GC-rich sequence in the multiple cloning site 3' to the T7 promoter of the pGEMfos/NHc (Figure 1); this probably resulted in the formation of secondary structures at the 5' terminus of the *c-fos* RNA probe, which was resistant to RNAase digestion.

Taken together, these results indicate that *c-fos* and butyrate both stimulate TRE response genes; furthermore, *c-fos* is likely to act in the downstream signalling pathway of butyrate.

Figure 5 Morphologies of F-98 cells and stable fos-expressing clones

(a, b) Control F-98 cells; (c, d) clone 1-9; (e, f), clone 1-15; (g, h), clone 1-17; (i, j) clone 3-4; (k, l) clone 3-5. (a, c, e, g, i, k) Exponentially growing cells; (b, d, f, h, j, l) confluent cells. Cells were photographed by phase-contrast light microscopy.

Isolation and characterization of stable *c-fos*-expressing clones

To delineate further the role of *c-fos* in F-98 cells, a plasmid containing mouse *c-fos* gene, under the control of the RSV promoter (pRSVfos/NaeI), was co-transfected with pSV2neo, the geneticin-resistant plasmid, into F-98 cells. Stable transfectants were selected for the ability to replicate in medium containing G-418. It was noted that in the presence of *c-fos* expression vector the number of colonies decreased, and the proportion of small-sized colonies (diameter < 3 mm) increased considerably when compared with transfection studies using the vector control. As shown in Table 3, in two representative

Figure 6 Proliferation of the *fos*-expressing and non-*fos*-expressing cells

Cells were seeded at a density of $1 \times 10^5/60$ mm plate; medium was changed every other day. Various *fos*-expressing and non-*fos*-expressing cells were counted at the indicated times. Key: F-98, control F-98 cells; 1-9, transfected F-98 cells without *c-fos* mRNA expression; 1-15, 1-17, 3-4 and 3-5 are cell clones with *c-fos* expression.

Table 4 Growth properties of *fos*-transfected clones

F-98 cells were stably transfected with pRSV*fos*/NaeI and pSV2neo and G418-resistant clones were isolated as stated in the Materials and methods section. Evidence of *fos*-mRNA expression in these cells was analysed by RNAase-protection analysis.

Clone	<i>fos</i> expression	Serum dependency*	Saturation density†	Anchorage dependency‡
F-98	—	—	High	—
1-9	—	—	High	—
1-15	+	+	Low	—
1-17	+	+	Low	—
3-4	+	+	Low	—
3-5	+	+	Low	—

* Cells (5×10^5) were plated in a 60 mm-diameter tissue-culture dish. The cells were switched to serum-free medium after 24 h and the viability of cells was observed under a microscope after 10 days: '+' indicates that cells depend on serum for survival, whereas '—' indicates that cells proliferate in the absence of serum.

† Cells (5×10^5) were plated in a 60 mm-diameter tissue-culture dish, and medium was changed every other day. After 10 days the cell numbers were obtained. 'Low' indicates that cell numbers are less than 2×10^6 . 'High' indicates that cell numbers are greater than 2×10^7 .

‡ Cells (1×10^5 cells/1.5 ml of medium) were mixed with an equal volume of 2.6% methylcellulose and layered on a 0.9%-agar plate. After 10 days, colonies were scored under a microscope: '—' indicates a high level of colony formation.

experiments, the average number of total colonies fell from 246 to 107 in the presence of *c-fos* expression; furthermore, among the 107 colonies obtained, the proportion of small colonies increased from 38% (93/246) to 48% (51/107). These findings suggest that over-expression of *c-fos* may suppress colony formation.

Some 34 clones were selected from the smaller-sized colonies for examining the expression of *c-fos* mRNA. RNAase-protection assays indicate the presence of *c-fos* transcripts in many of these isolated clones (results not shown). Clones containing transfected *c-fos* were randomly selected for further characterization. These cells, in general, showed altered morphology; instead of the epithelioid shape of the parental F-98 cells, they assumed different and various morphologies (Figure 5). The growth rate also decreased considerably; as indicated in Figure 6, transfected cells not expressing *c-fos* (clone 1-9) or control F-98 cells exhibited faster doubling times and grew to higher

saturation densities than those of the *c-fos*-expressing transfectants (clones 1-15, 1-17, 3-4 and 3-5). In addition, in clear contrast with F-98 cells or transfectants that did not express *c-fos* (clone 1-9), all of the four *c-fos*-expressing transfectants showed serum-dependent growth. Since glial or glioma cells are known to form colonies in semi-solid culture [38], the anchorage-dependent growth assay therefore could not distinguish differences between constitutive *fos*-expressing clones from non-*fos*-expressing cells. All cells analysed showed the ability to form colonies in semi-solid culture. These results are summarized in Table 4. These data provide evidence that *c-fos* expression changed the morphology and altered the growth characteristics of F-98 glioma cells.

F-98 cells express GFAP, a specific marker for glial cells; previous studies indicated that levels of GFAP could be further elevated by butyrate treatment. We next examined whether the *fos* transfectants had an increased GFAP expression. As shown in Figure 7, immunofluorescent staining with monoclonal anti-GFAP antibody indicates that the GFAP expression in two *c-fos* transfectants (clones 1-15 and 1-17) was significantly elevated over that of the control F-98 cells or non-*fos*-expressing transfectant (clone 1-9). These results suggest that *c-fos* expression may have an important role in glial-specific marker expression.

The *c-fos* 5'-regulatory region confers butyrate response

The possibility of the presence of DNA sequences in the *c-fos* upstream regulatory region that mediated the butyrate induction of *c-fos* gene activation was examined. For this purpose, different segments of the human *c-fos* promoter were linked to the CAT reporter gene (Figure 1b) and the constructs transfected into F-98 cells. The ability of butyrate to induce the CAT activity was then analysed. As shown in Table 5, a moderate but reproducible induction of CAT activity was observed after butyrate exposure in cells transfected with pFC2 (−1400 to +42) and pF750 (−757 to +42). These results indicate that the sequence within the proximal −757 bp of the *c-fos* gene conferred the butyrate effect on *c-fos* gene activation. pFC9, which contained nucleotides −225 to +42, lacked response to butyrate activation.

On the other hand, pFCBS (−757 to −402), which lacks most of the currently known response elements in the *c-fos* gene, was able to respond to butyrate. These data suggest that the butyrate-response element may be located in the −757 to −402 region of the *c-fos* gene.

DISCUSSION

Although stimulation of the *c-fos* gene was indicated in many differentiating cells, evidence to point to the precise role for its expression is rather scarce. Müller and Wagner [39] have shown in *c-fos*-transfected F9 teratocarcinoma stem cells that the morphology and expression of several specific markers were characteristic of differentiated cells, implying that *c-fos* played a role in differentiation. On the contrary, it has been demonstrated that *c-fos* expression is neither sufficient nor obligatory for the differentiation of human U-937 or HL-60 leukaemia cells [15]; by following the kinetics of *c-fos* and *c-jun* gene-family-member expression, Szabo et al. [40] have revealed a tight correlation between *c-jun* expression, the generation of AP-1 activity, and the differentiation of cells, suggesting a critical role for *c-jun*, and not *c-fos*, in the differentiation of these cells. In the case of NGF-induced differentiation of PC12 cells, although *c-fos* activation was demonstrated, the *fos*-expressing PC12 cells blocked the differentiation induced by NGF [41]. These studies suggest a complicated relationship between *c-fos* expression and cellular differentiation.

Figure 7 Immunofluorescent staining of GFAP on *fos*- and non-*fos*-expressing cells

All cells were seeded on coverslips for 24 h. After fixation, the cells were first incubated with monoclonal antibody to GFAP (Dako Co.), and then incubated with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G antibody as described in the Materials and methods section. Fluorescein-labelled cells were observed under a fluorescence microscope (magnification $\times 400$). All photographs were taken with the same exposure time. (a) Control F-98 cells; (b) transfectant without *c-fos* expression (clone 1-9); (c) *c-fos* transfectant (clone 1-15); (d) *c-fos* transfectant (clone 1-17). Although GFAP staining is apparent in all cells, strong staining is observed in *fos*-transfected cells.

Table 5 The human *c-fos* promoter confers butyrate responsiveness

F-98 cells were transiently transfected with 7.5 μg of the designated plasmids (Figure 1b) plus 12.5 μg of pGEM3Zf+ as carrier DNA; 12–16 h later medium was refed. The cells were incubated with (+) or without (–) 2.5 mM sodium butyrate for 18 h, cell lysates were prepared and 10 μg of lysate proteins was assayed for CAT activity. CAT activity is expressed as pmol of acetylated chloramphenicol/h per mg cell lysate protein. Fold induction is defined as the ratio of CAT activity measured in the presence versus that in the absence of sodium butyrate treatment. Data are represented as means $\pm$ S.D. Numbers of independent transfections are indicated in parentheses.

Plasmid transfected	CAT activity (pmol/h per mg)		Fold induction
	–	+	
FC2	10935 $\pm$ 5349	26870 $\pm$ 10475	2.49 $\pm$ 0.37 (6)*
F750	7543 $\pm$ 3151	15324 $\pm$ 5313	2.30 $\pm$ 0.01 (6)†
FC9	3027 $\pm$ 185	3363 $\pm$ 284	1.11 $\pm$ 0.04 (5)‡
FCBS	4593 $\pm$ 583	9485 $\pm$ 759	2.29 $\pm$ 0.17 (8)§

* $P < 0.02$, † $P < 0.02$, ‡ $P > 0.1$, § $P < 0.001$, suggesting that, except for FC9, significant stimulation by butyrate is seen with FC2, F750 and FCBS.

homodimer and activates transcription, the Fos–Jun complex has a higher binding constant and trans-activation activity toward DNA [42,43]. It is therefore conceivable that, once induced by butyrate, Fos can co-operate with the pre-existing Jun and trans-regulate many secondary-response genes. We have shown in the butyrate-treated cells that Fos is participating in the binding to TRE oligonucleotide. The functional significance of Fos expression is evidenced by transfection studies using the plasmid construct of the CAT gene under the control of TRE. Exposure to butyrate or co-transfection with *c-fos* results in increased CAT activity (Tables 1 and 2). Furthermore, the ability of butyrate to activate CAT expression is greatly diminished in the presence of *c-fos* over-expression. The finding that there is no additive effect on CAT stimulation by the combined actions of Fos and butyrate suggests that Fos and butyrate activate the TRE-driven gene expression through the same pathway. Since butyrate induces c-Fos expression, it is likely that Fos is a downstream mediator of butyrate in activating the TRE-response genes. Further proof for the involvement of *c-fos* in butyrate action may require methods that effectively abolish *c-fos* expression.

The phenotypic changes in the *c-fos*-expressing cells indicate that Fos expression induced alteration in morphology and changed growth properties, including serum dependency, contact inhibition, and proliferation rate of F-98 cells. Furthermore,

We have shown that c-Jun was constitutively expressed, whether or not butyrate was present. Although Jun forms a

increased expression of a glial-specific protein, GFAP, was noticed. These changes, accompanied with Fos expression, correlate with a more differentiated phenotype. That *c-fos* expression may be associated with cellular differentiation was recently suggested for Fos transgenic mice. It was shown that sites of constitutive Fos expression did not associate with cellular proliferation; instead, a correlation between *c-fos* expression and cells undergoing a terminal differentiation process was noted [44]. Although we have shown that *c-fos* expression inhibits the growth and changes the morphology of F-98 cells, at present we have no evidence that these effects are connected to the butyrate-induced alterations in F-98 cells.

Our results indicate that the sequence responsible for *c-fos* activation is located at nucleotides -757 to -402 of the *c-fos* gene (Table 5). Until now, the enhancer elements identified on the *c-fos* promoter were all localized within the -400 nucleotides upstream of the cap site; the -400 to -200 region, which contains multiple cis-acting elements, was also highly conserved between human and mouse *c-fos* genes [33]. It appears that butyrate does not act through these known response elements to enhance *c-fos* transcription. Our finding of a butyrate-response element distal to this region suggests that the *c-fos* gene is under a more complex array of regulation than those currently understood. Sequence comparison between human and mouse *c-fos* promoters indicates that regions between -400 and -757 share little similarity, except near position -744, where the human sequence TCTGCTTCCA is identical with that of mouse sequence at -734, and that the sequence TGCCTGAATTA located at -660 of the human gene is similar to that of mouse gene at -640. Previous studies [37] indicate that butyrate was able to activate RSV promoter; comparison between human *c-fos* and RSV promoters showed similarity at around position -660 of the human *c-fos* gene. Further study is required to identify clearly the exact sequence in the *c-fos* gene responsible for the butyrate effect.

Butyrate has been reported to activate several viral promoters, including SV40 [37], HIV-1 [45,46] and cytomegalovirus [47], and cellular genes such as the embryonic globin gene [48], the gonadotropin α gene [49] and the proenkephalin gene [50]. It is possible from the present findings that some of the activation could be due to immediate early gene induction, or that butyrate could act through an unidentified transcription factor such as its induction of *c-fos* expression. In butyrate activation of the HIV-1 promoter, it appeared that transcription factor Sp1 was involved, since mutation on the Sp1 binding site diminished the butyrate effect [45]. Tanaka et al. [47] reported that the enhancing effect of sodium butyrate on human cytomegalovirus replication may be mediated through some host cellular factor which activates the virus's major immediate early gene (IE1) promoter. In contrast with the present findings that butyrate was able to activate *c-fos* expression, Saito et al. [51], using different human hepatoma cell lines, demonstrated that sodium butyrate exhibited a differential effect on several oncogenes; in HCC-M hepatoma cells, *c-fos* expression was inhibited. These findings suggest that factors mediating the butyrate effect on *c-fos* gene expression may be under cell-specific regulation.

Butyrate has been shown to induce differentiation and growth inhibition in a variety of neoplastic cell types; however, the molecular mechanism of its action remains speculative. We have presented evidence in this paper that, in F-98 cells, butyrate induces *c-fos* expression through sequences upstream of the 5'-regulatory region and trans-activates TRE-response genes. Moreover, constitutive expression of the *fos* proto-oncogene plays a critical role in regulating growth and differentiation of F-98 glioma cells.

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