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REGULATION OF C-FOS EXPRESSION BY SODIUM BUTYRATE IN THE HUMAN COLON CARCINOMA CELL LINE CACO-2

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Summary: We used sodium butyrate to modify the differentiation and growth properties of the Caco-2 colon adenocarcinoma cell line and considered c-fos proto-oncogene expression as a potential target. C-fos is induced by butyric acid very rapidly at a post-transcriptional level and is stimulated transcriptionally at later times. This transcriptional induction does not result in an increase in steady-state mRNA levels. We show by transient transfection assays that the ATF-CRE binding site located between -63 and -54 relative to the c-fos transcriptional start site is a target for butyrate-induced fos transcription. Furthermore, gel retardation assays show an increase in CRE binding activity in cells treated with butyrate. These results demonstrate that butyrate can affect specific transcription factors important for cell growth and differentiation at multiple levels of regulation.

The human colon carcinoma cell line Caco-2 (1) spontaneously undergoes enterocytic differentiation in culture (2). This cell line represents an in vitro model for the analysis of intestinal cell differentiation functions (3-6).

In order to study differentiation of cells in culture, polar organic solvents, such as butyric acid, have been used to modify growth and differentiation properties (7). Butyric acid, a four-carbon fatty acid, is a natural fermentation product of the colon microflora (8,9). It induces histone hyperacetylation by inhibiting histone deacetylase and selectively inhibits phosphorylation of histones H1 and H2A (7). Butyric acid elicits a broad spectrum of responses in cultured cells, namely blocking DNA synthesis and altering cell morphology, growth rate, enzymatic activities and expression of a variety of mammalian genes. Butyric acid affects expression of many genes in colon carcinoma cell lines: in some lines, the carcinoembryonic antigen gene (CEA) is induced (10), and in most, a placental-type alkaline phosphatase is activated (11,12). No correlation has been found between changes in nuclease sensitivity as a result of butyrate treatment, and induction of gene expression (13,14). However, recent reports suggest that butyric acid can modulate gene expression through specific promoter regions. For instance, transcription of herpes simplex immediate early genes is induced by butyrate in neuroblastoma cells (15). A 156 bp fragment in the promoter region of the chicken embryonic globin gene was found to be necessary for butyrate dependent activation in mouse erythroleukemia cells (16). Recently, sequences containing Sp1 transcription

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binding sites were found to be part of a butyrate-inducible region in the human immunodeficiency virus (HIV) type I long terminal repeat (17).

Since butyrate affects gene expression in many cell types, including colon carcinoma cell lines, we considered c-fos proto-oncogene expression as a potential target for butyric acid in the Caco-2 cell line. Activation of c-fos is part of the cellular response to a wide variety of extracellular stimuli (18). The c-fos gene product dimerizes with the c-jun protein to form a complex that recognizes the AP-1 or TPA-responsive element (TRE), enabling this transcription factor to regulate transcription from nearby promoters (19). We show here that butyrate controls c-fos expression at post-transcriptional and transcriptional levels. We have identified the activating transcription factor (ATF)- cyclic AMP response element (CRE) binding site located between - 63 and -54 relative to the fos start site as a specific target for fos butyrate-dependent transcriptional activation.

Materials and methods

Cell culture: The human enterocyte-like cell line Caco-2-15 used as a model to study intestinal cell differentiation and function in vitro was obtained from A. Quaroni (Cornell University, Ithaca, NY). This clone of the parent Caco-2 cell line (HTB 37; ATCC, Rockville, MD) has been characterized elsewhere (20). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). 24 h prior to sodium butyrate treatment, medium was changed to DMEM without serum. Sodium butyrate (Sigma), at a 5 mM final concentration, was added to cells at 70-80% confluency.

DNA transfections and transient expression assays: Reporter plasmids were introduced in Caco-2 cells by using the calcium phosphate-mediated transfection of adherent cells in suspension (21). Briefly, after trypsinization and centrifugation, cells were resuspended in 1 ml of calcium phosphate precipitate containing 10 μg of plasmid DNA. The calcium phosphate-DNA suspension was mixed with DMEM containing 0.5% FBS and equal amounts of cells and precipitate were plated for 24 h in four 60 mm dishes. Two plates out of four were treated with sodium butyrate. Cells were harvested and lysed 10 h after treatment with 5 mM sodium butyrate. After normalization of samples for protein concentration, cell extracts were incubated with [^{14}C]- chloramphenicol and assayed for chloramphenicol acetyltransferase (CAT) activity as described previously by Gorman et al. (22).

RNA isolation and analysis: Cytoplasmic RNAs were prepared by the urea method (23) and denatured with formaldehyde for Northern blot analysis (24). Hybridizations were performed with the following probes: a 658 bp PstI-PvuII human c-fos fragment (25); an EcoRI fragment of the human β-actin gene (26).

Nuclear run-on assays: Cells were harvested in phosphate-buffered saline, centrifuged and lysed in 0.5 ml of buffer A (27) containing 0.25% NP-40. After centrifugation, the supernatant was recovered for cytoplasmic RNA purification. The nuclei were resuspended in nuclei storage buffer (27) containing 100 U of RNasin (Promega) per ml. Nascent transcripts were elongated in vitro for 30 minutes at 26°C and run-on analysis was done as described previously (28). The nuclear RNA was hybridized to the following DNAs: fos, a 658 bp PstI-PvuII human c-fos fragment (25); GAPDH, a PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase gene (29). Signal intensity was quantitated by densitometry with a Pharmacia LKB XL Ultroscan.

Plasmids and oligonucleotides: The fos-CAT constructs pFC700, pFC225, pFCΔ94/61 and pFCΔ94/72 have been described previously (30). pFC700 contains the wild-type c-fos promoter linked to the CAT gene. In pFC225, deletion of c-fos promoter sequences between -700 and -225 removes the serum response element (SRE, -317 to -298) and an AP-1 binding site (-303 to -283). pFCΔ94/61 has a deletion of the proximal promoter region between -94 and -61: this deletion removes two direct repeats, a MLTF site and an ATF-CRE recognition site important for c-fos expression. pFCΔ94/72 differs from the latter by the presence of the ATF-CRE site (30). An oligonucleotide corresponding to the cyclic AMP responsive element sequence (CRE-ATF) (5'TGACGTCATGACGTCA-3') was inserted in the BamHI site of a derivative (-37TKCAT) of the thymidine kinase-CAT vector pBLCAT2 (31) with a deletion of the thymidine kinase promoter sequences between -105 and -37. This CRE-ATF oligonucleotide and the AP-1 binding site (5'GTGAGTCAGTGACTCA-3') were used for the gel retardation assays.

Extract preparation and gel retardation assays: Crude nuclear extracts were prepared according to Stein et al. (32). Briefly, 10^7 cells were harvested in phosphate-buffered saline and suspended in 100 μl of lysis buffer (32). After 5 minutes on ice, nuclei were recovered after centrifugation at 9,000xg for 30 seconds. The nuclei were then washed in 300 μl of lysis buffer without NP-40 and suspended in 100 μl of nuclear resuspension buffer (32). Nuclei were lysed by three freeze-thaw cycles and extracts were obtained after centrifugation at 15,000xg for 15 minutes. Gel retardation assays were done as described previously (33,34). Samples were electrophoresed in a 4% polyacrylamide gel in 0.5% Tris-borate buffer.

Results and discussion

Cells, at 80% confluency, were treated with 5mM sodium butyrate without serum. Northern analysis of cytoplasmic RNAs isolated from cells treated for different times with butyrate shows a rapid increase in c-fos mRNA levels after 30 minutes (Fig. 1a). These induced levels remained constant, as long as butyrate was present (Fig. 1a, data not shown). This was determined by comparison to an β-actin control after densitometric analysis.

Next, we verified by a run-on transcription assay if butyrate was affecting c-fos expression at the level of transcriptional initiation. Nuclei from butyrate treated cells were isolated and RNAs were elongated *in vitro* (28). Equal counts of nascent radioactively labeled transcripts were hybridized to c-fos and GAPDH DNA fragments immobilized on nitrocellulose filters. The data show that butyrate induces transcription of the c-fos gene late in time, after a 2 hour delay (Fig. 1b). After 4 h, c-fos transcription is increased more than 3.5-

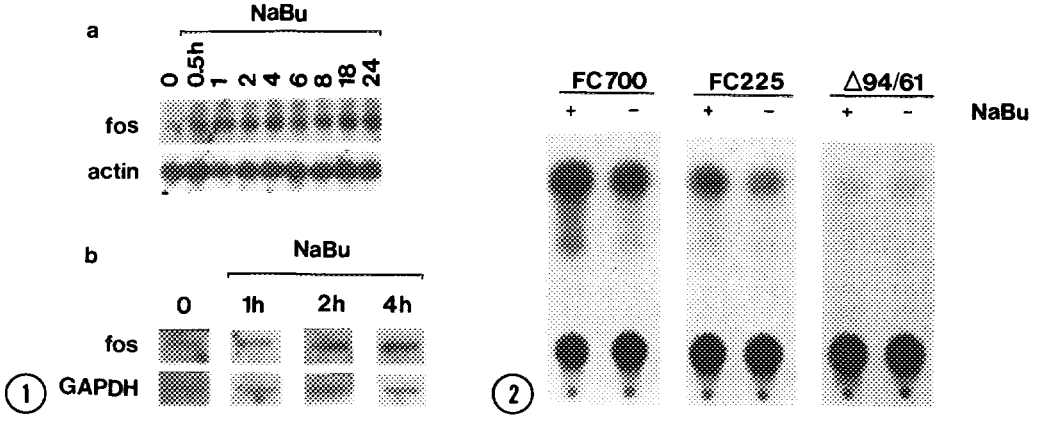

Figure 1. a.Induction of c-fos expression by butyrate. Cytoplasmic RNAs were isolated from Caco-2 cells treated with 5 mM sodium butyrate in the absence of serum for 30 minutes to 24 hours, as indicated. RNAs were separated and hybridized to a c-fos specific RNA probe. The filter was rehybridized to a human β-actin DNA probe, as a control for RNA loading. b.Transcriptional activation of c-fos by butyrate. Nuclear run-on assays were performed as described previously (28). Nuclei were isolated from Caco-2 butyrate-treated cells at the indicated times (0, 1, 2 and 4 hours). The fos probe is a 658 bp PstI-PvuII human c-fos fragment (25) and GAPDH is a PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase gene (29).

Figure 2. C-fos upstream promoter sequences between -94 and -61 are essential for butyrate-dependent c-fos activation. The c-fos promoter (pFC700) and deletion mutants (pFC225, Δ94/61), linked to the CAT reporter gene were transfected in Caco-2 cells in the absence (-) or presence (+) of 5 mM sodium butyrate (10 hour treatment). Cell extracts were assayed for CAT activity (22). Acetylated and unacetylated forms of chloramphenicol were separated by thin-layer chromatography. Equivalent amounts of protein were used in each assay. These experiments were repeated six times.

fold, as measured by densitometry and comparison to a GAPDH control. This means that post-transcriptional regulation mechanisms account for the rapid early induction after 30 minutes. Post-transcriptional mechanisms are also involved at later times since a 3.5-fold increase in c-fos transcription does not result in a parallel increase in steady-state mRNA levels.

To assess the role of various c-fos promoter sequence elements in butyrate-dependent transcriptional activation, plasmids, containing the normal c-fos promoter (pFC700), or deletions of defined sequences important for c-fos transcription and linked to the CAT gene, were introduced in Caco-2 cells by the calcium phosphate transfection method (21). Serum-starved transfected cells were treated with 5mM sodium butyrate for 24 hours. Cytoplasmic extracts were prepared and assayed for CAT activity, which was taken as a measure of transcriptional activity. Fig. 2 shows that butyrate indeed stimulates transcription from the c-fos promoter (see pFC700) 2.6 fold above the constitutive level of expression, as measured by scanning densitometry ($p<.005$). Deletion of the upstream elements (pFC225) does not significantly alter butyrate-induced c-fos activation (2.4 fold). These data show that the region between -700 and -225, which includes the SRE and AP-1 transcriptional regulators, is not a target for butyrate. However, deletion of the proximal -94 to -61 region, important for basal and induced c-fos transcription (30,35), abolishes butyrate-dependent induction (Fig. 2, pFCΔ94/61).

To determine whether the direct repeats or the ATF-CRE site deleted in the pFCΔ94/61 construct were necessary for c-fos induction by butyrate, we compared the transcriptional activity of this mutant and deletion mutant pFCΔ94/72, which retains the ATF-CRE binding site. Although this deletion reduces basal transcription (30,35), the presence of the ATF-CRE binding element restores a 2-fold c-fos stimulation by butyrate (Fig. 3;$p<.005$). These data suggest that the ATF-CRE region may act as a butyrate-response element.

To make sure that the ATF-CRE binding site is a specific target, we inserted a synthetic ATF-CRE site upstream of a minimal thymidine kinase promoter linked to the CAT gene (31). CAT assays (Fig. 3) show that butyrate stimulates transcription from a promoter containing one or two copies of the ATF-CRE element. These data confirm that the ATF-CRE region is important for butyrate-dependent transcriptional activation.

Since c-fos is transcriptionally induced through a specific ATF-CRE binding site, we asked whether the ATF-CRE binding activity was enhanced in butyrate treated cells. In a gel mobility shift assay, nuclear extracts were mixed with ATF-CRE or AP-1 labeled oligonucleotides. The resulting complexes were separated from the free probe on a native polyacrylamide gel. Fig. 4 shows that an ATF-CRE binding activity is in fact induced by butyrate. Interestingly, butyrate does not induce AP-1 binding activity (Fig. 4).

Little is known about the modulation of specific genes by butyric acid and just how these alterations in gene expression lead to cellular modifications. In this report, we have shown that butyrate regulates c-fos in the Caco-2 colon carcinoma cell line at both post-transcriptional and transcriptional levels. Post-transcriptional mechanisms of regulation are responsible for rapid c-fos induction and for constant steady-state mRNA levels even after a 3.5-fold increase in transcription. This gene is part of a subset of genes whose transient accumulation is due first to their brief activation and to the extreme

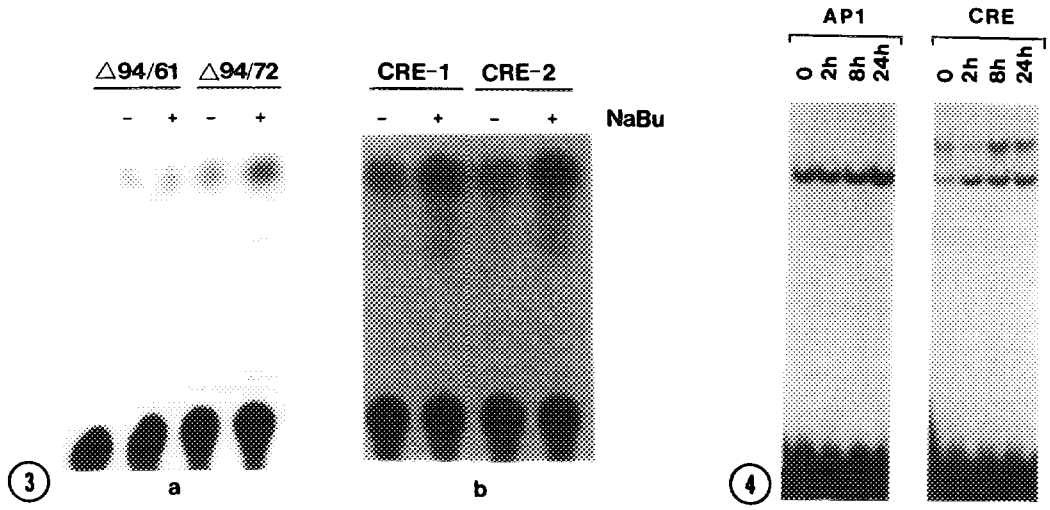

Figure 3. The c-fos ATF-CRE element is essential for butyrate-dependent c-fos activation. The c-fos promoter deletion mutants with (Δ94/72) or without (Δ94/61) the ATF-CRE element, and constructs containing one (CRE-1) or two (CRE-2) copies of a CRE oligonucleotide inserted upstream of a minimal promoter (31) and linked to the CAT gene, were transfected in Caco-2 cells in the absence (-) or presence (+) of 5 mM sodium butyrate (10 hour treatment). Cell extracts were assayed for CAT activity (22). Equivalent amounts of protein were used in each assay. These experiments were repeated six times.

Figure 4. Effect of butyrate on ATF-CRE and AP-1 related binding activities. Nuclear extracts from cells treated with butyrate for 0, 2, 8 and 24 hours were prepared (32) and mixed with ATF-CRE or AP-1 sequence labeled probes. DNA-protein complexes were separated from the free probe on a native polyacrylamide gel as described previously (33,34).

instability of their mRNA transcripts (36). Butyrate could alter c-fos mRNA stability at early and late times after induction in a temporal and transcription-dependent manner. This could reflect modulation in the synthesis or post-translational modification of polypeptides normally involved in degradation or stabilization of c-fos mRNAs, such as proteins binding to the AU-rich sequence in the 3'-untranslated region of c-fos (37).

Addition of cycloheximide, a protein synthesis inhibitor, to butyrate treated cells generated a very strong transient superinduction of c-fos mRNA levels, as seen in other systems (18,36). Elevated mRNA levels remained constant afterwards and were dependent on a continuous transcriptional activation induced by cycloheximide (data not shown). This cycloheximide effect on fos transcription prevented us from determining whether butyrate was modifying pre-existing proteins or was needed to activate gene expression. Other investigators have shown that cycloheximide repressed butyrate-induced transcription from the Moloney murine sarcoma virus enhancer (38). The butyrate-dependent germ cell alkaline phosphatase gene activation (39) and c-myc proto-oncogene repression (40, Souleimani and Asselin, unpublished results) were suppressed by cycloheximide. It has been suggested that butyrate mediates its effects through induction of new protein synthesis. However, cycloheximide does not repress butyrate induction of the metallothionein or vimentin genes (41,42). These data imply that butyrate could act directly, by inducing protein modifications. Recently, it has been shown that butyrate inhibits myogenesis by interfering with

the transactivating properties of MyoD and myogenin (43). Thus, both levels of regulation could be involved.

We identified a specific butyrate-responsive element in the c-fos promoter as an ATF-CRE binding site. The ATF-CRE site is one of the multiple elements mediating induction by cyclic AMP in the c-fos promoter (44,45). Since this element conveys, by itself, c-fos induction by butyrate, this indicates that butyrate does not affect cyclic AMP levels. The CREB protein which recognizes the c-fos ATF-CRE site (46) is a nuclear phosphoprotein dimer. Phosphorylation can regulate dimerization, binding activity and transcriptional efficiency of the CREB protein in vitro (46). Thus, increases in binding activity could result from increased expression of some members of the ATF-CREB family of transcription factors, or from post-translational modifications affecting DNA binding (47). The fact that AP-1 binding activity is not increased confirms that butyrate affects c-fos transcription through specific targets. Butyrate's effect on particular families of transcription factors could explain its ability to regulate a small subset of genes involved in growth and differentiation (48).

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