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Regulation of c-*myc* expression by sodium butyrate in the colon carcinoma cell line Caco-2

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The human colon carcinoma cell line Caco-2 spontaneously undergoes enterocytic differentiation in culture. We used sodium butyrate to modify differentiation and growth properties of this cell line and considered c-*myc* expression as a potential target. Degradation of normal c-*myc* mRNAs with a half-life of 20 min is not coupled to translation in this cell line, as determined by cycloheximide treatment. We show that butyrate reduces c-*myc* mRNA levels after a 30 min delay. Butyrate does not affect c-*myc* expression at the level of transcriptional initiation or elongation, as determined by run-on analysis, but at a post-transcriptional level. Cycloheximide blocks butyrate-dependent reduction of c-*myc* mRNA levels. Cross-linking experiments show that a 34 kDa protein binds specifically to the c-*myc* AU-rich instability determinant found in the 3′-untranslated region (ARE). Binding of this protein to the ARE is not modulated by butyrate or cycloheximide. These experiments suggest that butyrate induces a factor involved in c-*myc* mRNA degradation that differs from the known ARE-associated proteins. Post-transcriptional modification of gene expression could be one of the major targets for this anti-proliferative agent.

c-*myc*; Butyrate; Caco-2; Cross-link; mRNA stability; Run-on

1. INTRODUCTION

The human colon carcinoma cell line Caco-2 [1] spontaneously differentiates into enterocytes in culture [2]. Under standard culture conditions, Caco-2 cells form confluent monolayers consisting of well polarized columnar cells with tight junctions and a typical apical brush border [3]. Hydrolases associated with the small intestinal brush border, such as sucrase-isomaltase, lactase, and aminopeptidase are also expressed [4].

Polar organic solvents, such as butyric acid, a four-carbon fatty acid, have been used to modify the growth and differentiation properties of cells in culture [5]. Butyric acid is a natural fermentation product of the colon microflora [6,7]. Butyric acid treatment of cultured cells creates major modifications in chromatin structure and cytoskeletal assembly, and alterations in cell morphology, growth rate, DNA synthesis, enzymatic activities and expression of different mammalian genes without affecting the level of protein synthesis [5]. In colon carcinoma cell lines, butyric acid induces expression of the carcinoembryonic antigen gene (CEA) [8] and of a placental-type alkaline phosphatase [9]. It was suggested that butyrate-induced gene expression was caused by alterations in chromatin structure through histone acetylation, as measured by the increased sensitivity of chromatin to DNase I digestion [10]. However, no correlation has been found between changes in nuclease sensitivity and induction of gene expression, as a result of butyrate treatment [11].

Recent reports have suggested that butyric acid can modulate gene expression through specific promoter regions. In murine erythroleukemia cells, a 156 bp promoter fragment was found to be necessary for butyrate dependent activation of the chicken embryonic globin gene [12]. Sequences containing Sp1 transcription factor binding sites were defined as a butyrate-inducible region in the human immunodeficiency virus (HIV) type I long terminal repeat [13].

In order to understand the effects of butyrate on proliferation and gene expression, we considered c-*myc* proto-oncogene expression as a potential target for butyric acid in the colon carcinoma cell line Caco-2. C-*myc* gene expression has been linked to the control of cell proliferation, differentiation and apoptosis [14]. This transcription factor, which forms an heterodimer complex with the product of the *max* gene [15], is one of a number of cellular genes whose expression is rapidly induced during the G0 to G1 transition by multiple mitogenic stimuli, independent of de novo synthesis [14]. C-*myc* expression is rapidly down-regulated after induction of differentiation in many cell types [14,16]. We show here that c-*myc* mRNA turnover in Caco-2 cells is independent of translation and that butyrate

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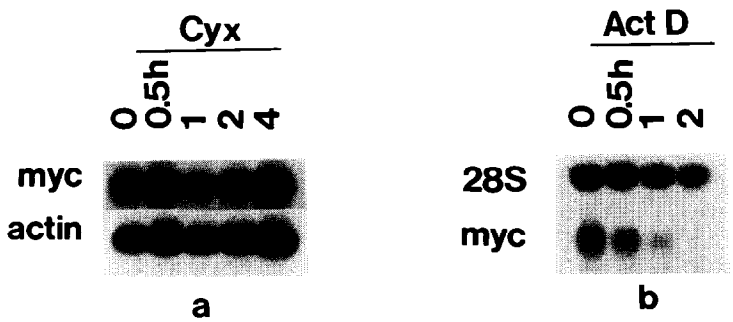

Fig. 1. (a) Effect of cycloheximide (Cyx) on c-*myc* mRNA steady-state levels. Cytoplasmic RNAs were isolated from Caco-2 cells grown in medium without serum and treated for the indicated times (0–4 h) with 40 μg/ml Cyx. RNAs transferred to a nylon membrane were hybridized to a c-*myc* specific RNA probe. The filter was rehybridized to a human β-actin DNA probe, as a control for RNA loading. b. Half-life of c-*myc* mRNAs. Cytoplasmic RNAs were isolated from Caco-2 cells treated for the indicated times (0–2 h) with 10 μg/ml actinomycin D (Act D). RNAs were hybridized to a c-*myc* specific RNA probe and rehybridized to a 28S DNA probe, as a control for RNA loading. Half-life was measured by scanning densitometry.

reduces c-*myc* mRNA levels by a post-transcriptional mechanism requiring new protein synthesis.

2. MATERIALS AND METHODS

2.1. *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 derived from the parent Caco-2 cell line (HTB 37; ATCC, Rockville, MD) has been characterized elsewhere [17]. 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. Cycloheximide and actinomycin D (Sigma) were used respectively at concentrations of 40 and 10 μg/ml.

2.2. *RNA isolation and analysis*

Cytoplasmic RNAs were prepared by the urea method [18] and denatured with formaldehyde for Northern blot analysis [19]. Hybridizations were performed with the following probes: a 1.3 kb *Cla*I-*Eco*RI human c-*myc* fragment containing the third exon and the 3'-untranslated sequences [20]; a human β-actin *Hin*dIII-*Eco*RI fragment [21] and a human 28S ribosomal RNA probe (ATCC 77235).

2.3. *Nuclear run-on assays*

Cells were resuspended in phosphate-buffered saline, centrifuged and lysed in 0.5 ml of buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM HEPES (pH 7.5), 2 mM EDTA, 5 mM EGTA, 15 mM spermine, 0.5 mM spermidine, 14 mM mercaptoethanol, 0.25% Nonidet P-40) [22]. After centrifugation, the supernatant was recovered for cytoplasmic RNA purification. The nuclei were resuspended in nuclei storage buffer [22] containing 100 U of RNasin (Promega) per ml. Nascent transcripts were elongated in vitro for 30 min at 26°C with [α-^{32}P]UTP, and run-on analysis was performed as described previously [23]. The labeled nuclear RNAs were hybridized to the following DNAs: mycA, a 604 bp *Sma*I-*Pvu*II human c-*myc* fragment representing the first exon (103 U); mycB, a 1.3 kb *Cla*I-*Eco*RI human c-*myc* fragment containing the third exon and the 3'-untranslated region (199 U) [20]; GAPDH, a *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase gene [24]. Signal intensity was quantitated by densitometry with a Pharmacia LKB XL Ultroscan.

2.4. *UV cross-linking and label transfer*

A 1.3 kb *Cla*I-*Eco*RI human c-*myc* fragment encompassing the third exon and the 3'-untranslated region [20] was subcloned in the pSP72 vector (Promega). The plasmid was linearized either with *Nsi*I (*ΔAU*, 634 nt transcript) or *Ssp*I (containing the AU-rich region, 830 nt transcript). Linearized plasmids were transcribed in vitro with bacteriophage RNA polymerase T7 according to Promega instructions. Labeled RNA transcripts were produced by incorporation of [α-^{32}P]UTP. Unlabeled RNAs were transcribed in a similar fashion except that the labeled nucleotide was replaced by 500 μM UTP. Cytoplasmic extracts were prepared from untreated and butyrate-treated Caco-2 cells in a lysis buffer containing 10 mM HEPES (pH 7.9), 1 mM EDTA, 60 mM KCl, 1 mM dithiothreitol (DTT), 0.25% Nonidet P-40 and 1 mM phenylmethylsulfonylfluoride (PMSF). Nuclei were pelleted by centrifugation at 14,000 × *g* for 2 min. The supernatant was mixed to 0.5 volume of resuspension buffer containing 0.25 M Tris-HCl (pH 7.8), 60 mM KCl, 1 mM DTT, 1 mM PMSF [25]. Cytoplasmic extracts were immediately frozen in dry ice and stored at −70°C.

Cytoplasmic extracts (20 μg) were incubated at room temperature for 20 min with ^{32}P-labeled RNA in a buffer containing 1 mM $MgCl_2$, 0.25 mM DTT, 10 mM HEPES (pH 7.9), 5 μg tRNA and 5% glycerol in a total volume of 15 μl. The reaction mixtures were transferred into a 96-well culture dish and were cross-linked for 4 min at 254 nm with a UV DNA transfer lamp (Fotodyne). RNase A was added at a 1 mg/ml final concentration and reactions were incubated at 37°C for 15 min [26]. Samples were heated at 100°C for 3 min in an equal volume of loading buffer and separated by electrophoresis on a SDS-polyacrylamide gel. ^{32}P-labeled proteins were visualized by autoradiography.

3. RESULTS AND DISCUSSION

Sodium butyrate treatment of Caco-2 cells results in growth inhibition and cellular and genetic alterations that affect the normal differentiation pathways (data not shown). These dramatic effects on proliferation and differentiation by butyrate, also found in other colon carcinoma cell lines [8,9], led us to consider c-*myc* as a potential target for butyric acid. C-*myc* transient accumulation is due first to its transcriptional activation and to the extreme instability of the mRNA transcripts [14,16]. C-*myc* steady-state mRNA levels in Caco-2 cells are unaffected in medium with or without serum (data not shown). Since c-*myc* expression does not seem to

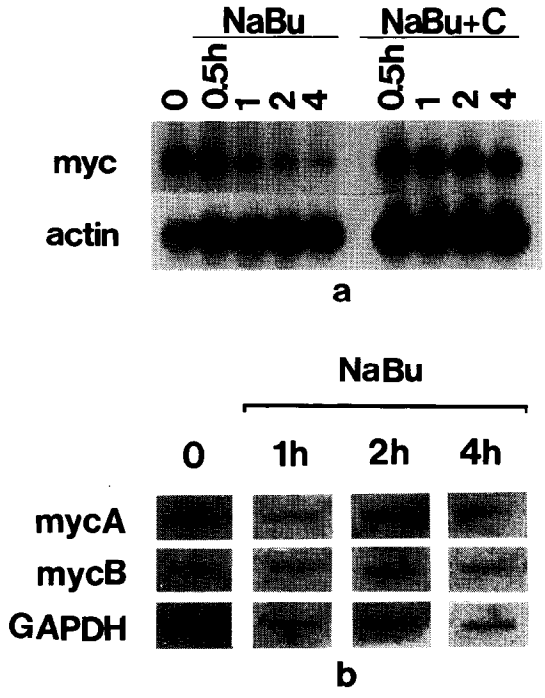

Fig. 2. (a) Effect of butyrate and cycloheximide on c-*myc* expression. Cytoplasmic RNAs were isolated from Caco-2 cells grown in medium without serum and treated for the indicated times (0–4 h) with 5 mM sodium butyrate alone (NaBu) or in combination with 40 μg/ml cycloheximide (NaBu+C). RNAs were hybridized to a c-*myc* RNA probe and rehybridized to a β-actin DNA probe, as a control for RNA loading. (b) Effect of butyrate on c-*myc* transcription. Nuclear run-on assays were performed as described previously [23]. Nuclei were isolated from Caco-2 butyrate-treated cells at the indicated times (1, 2 and 4 h). Labeled nuclear RNAs were hybridized to the following probes: mycA, a 604 bp *Sma*I-*Pvu*II human c-*myc* fragment containing first exon sequences [20]; mycB, a 1.3 kb *Cla*I-*Eco*RI human c-*myc* fragment encompassing the third exon and the 3′-untranslated region [20]; GAPDH, a *Pst*I fragment of the rat glyceraldehyde-3-phosphate dehydrogenase [24].

respond normally to serum components, we verified the effect of the protein synthesis inhibitor cycloheximide on its regulation. Inhibition of protein synthesis results in an increase in c-*myc* mRNA levels in most cell lines [16] because of translation-dependent control of mRNA stability [27–29]. Northern analysis of mRNAs isolated from Caco-2 cells treated with cycloheximide shows that c-*myc* mRNA levels do not increase in response to the inhibitor (Fig. 1a). This is consistent with results obtained with other colon carcinoma cell lines [30]. Half-life of c-*myc* mRNAs was measured after treatment of Caco-2 cells with the transcription inhibitor actinomycin D. Scanning densitometry shows a short half-life of 20 min (Fig. 1b). Thus, c-*myc* half-life is determined by mechanisms of stability unaffected by protein synthesis inhibitors in Caco-2 cells. This suggests that rapid c-*myc* mRNA degradation in this colon carcinoma cell line does not require translation of the mRNA, as opposed to its regulation in other cell lines [14,16,27–29].

Northern analysis of cytoplasmic RNAs isolated from cells treated for different times in medium without serum and containing 5 mM sodium butyrate shows an abrupt decrease in c-*myc* mRNA levels after a lag time of 30 min (Fig. 2a). These repressed levels remained constant, as long as butyrate was present, as determined by densitometry and comparison to the β-actin control (Fig. 2a, data not shown). Interestingly, addition of cycloheximide to butyrate-treated cells restored c-*myc* mRNA levels (Fig. 2a). To determine whether butyrate was modulating c-*myc* expression at the level of transcriptional initiation, we performed run-on transcription assays with nuclei from butyrate-treated Caco-2 cells. Equal counts of nascent radioactively labeled transcripts were hybridized to c-*myc* first exon (mycA), c-*myc* third exon and 3′-untranslated region (mycB), and GAPDH DNA fragments immobilized on nitrocellulose filters. Scanning densitometry with comparison to the GAPDH control shows that butyrate does not affect c-*myc* transcription at the level of initiation (see mycA, Fig. 2b). Furthermore, butyrate does not modulate the transcriptional block to elongation [14], as shown by comparison of signal intensities of mycA upstream and mycB downstream probes, and after taking into account the number of uridines in each probe (Fig. 2b). Scanning densitometry shows variations in the mycA:mycB ratio from 2.5 to 4.1 from one experiment to the other, with or without butyrate ($n = 4$). A weak block to elongation has also been found in the human colon carcinoma cell line COLO 320 HSR [31]. These data suggest that butyrate does not repress c-*myc* expression at the level of initiation or elongation. In fact, post-transcriptional mechanisms seem to account for the delayed reduction of c-*myc* mRNA transcripts. Butyrate could induce a nuclease or a protein targeting c-*myc* mRNAs to a degradation pathway other than the cycloheximide-independent pathway normally found in Caco-2 cells. This induction depends on new protein synthesis since cycloheximide, a protein synthesis inhibitor, restores the normal mRNA levels in the presence of butyrate.

Two distinct determinants have been implicated in the control of c-*myc* mRNA stability. One element consists of AU-rich sequences found in the 3′-untranslated region of the c-*fos* and c-*myc* proto-oncogenes, and of lymphokine genes [29,32–34]. These AU-rich elements (ARE) bind multiple proteins, including proteins of 32–37 kDa [35–37], and are not required in certain conditions for rapid c-*myc* mRNA degradation [38,39] because of the presence of other instability determinants. Interaction between the ARE and these proteins correlates with mRNA instability [35–37]. The second element localized in the third exon coding region [27,28] is a 182 nt fragment from the carboxy-terminal portion of the c-*myc* coding region which binds to a 75 kDa polysome-associated protein [40]. This sequence encodes a helix-loop-helix (HLH) domain implicated in

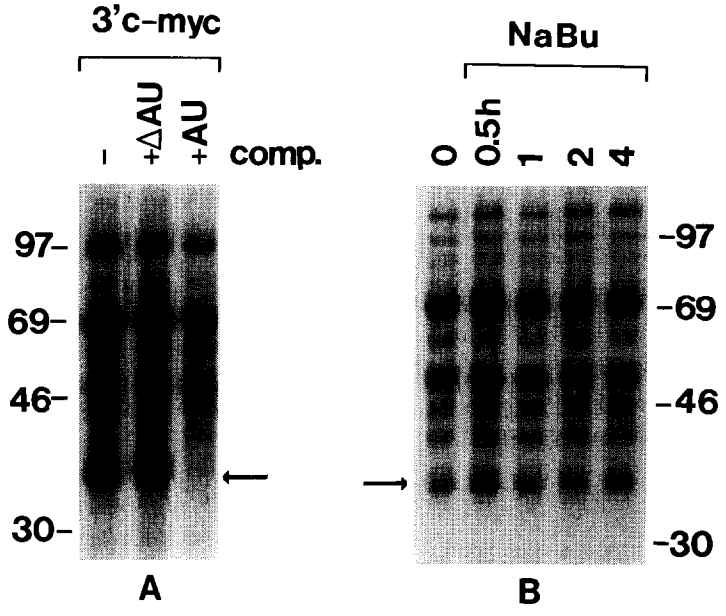

Fig. 3. UV-induced cross-linking of a 34 kDa protein to c-*myc* transcripts in vitro. RNAs transcribed in vitro with [α-^{32}P]UTP from a 830 bp *Cla*I-*Ssp*I c-*myc* fragment were incubated in vitro with Caco-2 cell cytoplasmic extracts and irradiated with UV light. Reaction mixtures were incubated with RNase A and separated on SDS polyacrylamide gel. (A) Radiolabeled c-*myc* RNA and 100-fold molar excess of either a 830 nt c-*myc* RNA containing the ARE (+AU) or a 634 nt transcript without the ARE (+ΔAU) were incubated with cytoplasmic extracts. (B) Radiolabeled c-*myc* RNA was incubated with cytoplasmic extracts obtained from cells treated for 0.5–4 h with 5 mM sodium butyrate (NaBu).

transcription factor dimerization [41]. It has been suggested that the 75 kDa protein protects this region from endonuclease attack [40]. To assess whether these proteins could be modulated by butyrate, thus explaining the increased instability of c-*myc* mRNAs in the presence of butyrate, we performed cross-linking experiments.

Cytoplasmic extracts were prepared from Caco-2 cells grown in medium without serum and were incubated with a ^{32}P-labeled riboprobe consisting of 830 nucleotides from the third exon and 3'-untranslated region of the c-*myc* gene [20]. To determine the specificity of this binding, 100-fold molar excess of unlabeled RNA with (+AU) or without (+ΔAU) AU-rich sequences between the *Nsi*I and *Ssp*I sites was used to compete for these complexes. The reaction mixtures were cross-linked by UV irradiation and incubated with RNase A. The labeled complexes were separated by electrophoresis on 10% SDS-polyacrylamide gels. Unlabeled c-*myc* RNA containing the AU-rich region efficiently competes for a 34 kDa protein present in Caco-2 cytoplasmic extracts (Fig. 3A). These data suggest that this factor binds specifically to AU-rich sequences in the c-*myc* 3'-untranslated region. The same or related proteins have been found to bind to ARE sequences in other genes [35–37].

Cytoplasmic extracts were prepared from Caco-2 cells treated with 5 mM sodium butyrate in medium without serum. The cross-linking experiment in Fig. 3B shows that butyrate does not modulate the RNA binding activity of the 34 kDa protein (Fig. 3B), nor does cycloheximide (data not shown). These data therefore suggest that reduction in c-*myc* mRNA levels caused by butyrate does not involve the 34 kDa protein associated with the AU-rich instability determinant.

Actinomycin D can block mRNA decay mediated by the c-*fos* ARE [33] but not by the c-*myc* ARE [28]. However, actinomycin D does in fact inhibit turnover mediated by the mRNA translation-dependent destabilizing element found in the c-*myc* third exon protein-coding sequence, in serum-stimulated cells [28]. Recently, You et al. [42] have observed a reduced binding activity of a 37 kDa protein to the c-*fos* ARE sequence after actinomycin D treatment. We thus verified if actinomycin D could affect the binding activity of the 37 kDa protein to the c-*myc* ARE. Cytoplasmic extracts were prepared as before from Caco-2 cells grown in serum-free medium with actinomycin D, in the presence or absence of butyrate. These extracts were incubated with the 830 nt labeled c-*myc* transcript, cross-linked and loaded on a SDS-polyacrylamide gel. Interestingly, actinomycin D, with or without butyrate, induced DNA binding activities of multiple proteins, including the 37 kDa protein and proteins of 88, 74, 44 and 41 kDa. Recently, Savant-Bhonsale and Cleveland [43] have presented evidence of a > 20S degradation complex implicated in mRNA instability through AU motifs for granulocyte monocyte colony stimulating factor mRNA. Inhibition of transcription by actinomycin D results in measured half-lives that cannot account for differences in RNA accumulation of certain mutants. They have suggested that transcription may be required for

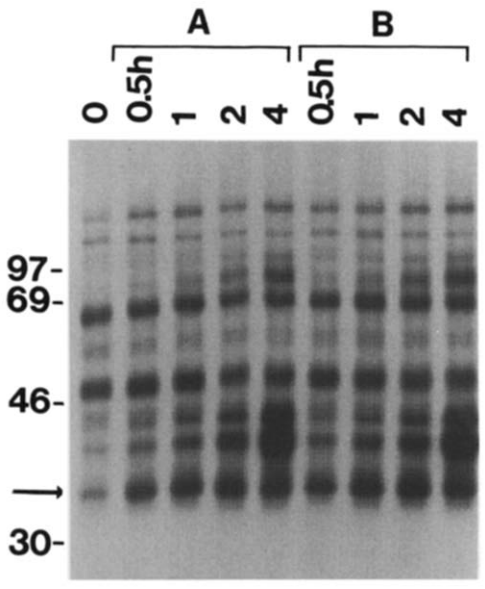

Fig. 4. Effect of actinomycin D on protein binding to c-*myc* transcripts in vitro. Cytoplasmic extracts were isolated from Caco-2 cells grown in medium without serum and treated with 10 μg/ml actinomycin D alone (A) or in combination with 5 mM sodium butyrate (B). Labeled transcripts from a 830 bp *ClaI-SspI* c-*myc* fragment containing the third exon and 3'-untranslated sequences were incubated with cytoplasmic extracts. Reaction mixtures were UV cross-linked, incubated with RNase A and fractionated on a SDS-polyacrylamide gel.

mRNA degradation because a short-lived RNA is involved directly or indirectly in the AU-dependent degradation pathway [43]. The labeled proteins appearing after actinomycin D treatment of Caco-2 cells could be bound to unstable RNAs. Degradation of these RNAs after transcriptional arrest would allow these proteins to interact with c-*myc* labeled RNAs. Proteins of 37, 66, 71 and 82 kDa have been found to interact with the c-*fos* ARE [37,42]. Proteins of 32–37 and 40 kDa interact with c-*myc* AU-rich sequences [35–37]. Whether these proteins are the same as those that bind c-*myc* RNA in response to actinomycin D in Caco-2 cells is not known.

We have shown here that c-*myc* mRNA turnover in a human colon carcinoma cell line is independent of translation since cycloheximide treatment does not result in an increase in steady-state mRNA levels, as seen in other systems [16]. Butyric acid, a known anti-proliferation and differentiation agent, causes an important decrease in steady-state c-*myc* mRNA levels after a 30 min delay. Translation inhibitor cycloheximide reverses butyric acid's effect however. Taken together, these results suggest that butyrate induces transcription of a factor involved in the control of c-*myc* expression. Alternatively, butyrate could induce small unstable RNAs part of a degradation complex. Since butyric acid does not affect c-*myc* expression at the level of transcriptional initiation or elongation, this factor probably regulates c-*myc* expression at a post-transcriptional level. The major post-transcriptional mechanism implicated in c-*myc* regulation occurs at the level of mRNA stability [14,16]. We have shown that butyrate does not affect the binding activity of a 34 kDa protein to c-*myc* AU-rich sequences (ARE) implicated in mRNA degradation. Butyric acid could induce a nuclease involved in mRNA degradation. Endonuclease attack could occur through the ARE or c-*myc* coding region determinants [28,35,40]. C-*myc* mRNA instability dependent on butyrate seems to be specific since c-*fos* mRNA levels are induced rapidly by butyrate at a post-transcriptional level (data not shown). The c-*myc* coding region determinant differs from the analogous element in c-*fos* by the presence of an HLH region and thus could be a target for butyrate dependent c-*myc* mRNA regulation.

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