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Direct Inhibition of the Expression of Cyclin D1 Gene by Sodium Butyrate

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In the mouse fibroblasts BP-A31 as well as in the human epidermoid carcinoma cells KB-3-1, both cyclin D1 mRNA and protein contents decreased rapidly during incubation with sodium butyrate. The decrease of cyclin D1 mRNA was not prevented by cycloheximide indicating that protein synthesis is not required for the inhibition of the expression of cyclin D1 gene by sodium butyrate. The 973 bp region upstream of the human cyclin D1 gene conferred inhibition of the expression of an indicator gene in transiently transfected cells. An 11 base-pair segment situated within this region, with a strong homology to the butyrate-response consensus element identified in butyrate-inducible promoters, also caused an inhibition of transcription under these conditions, indicating that cyclin D1 expression is inhibited by butyrate at the transcriptional level.

The progression of mammalian cells through G1 phase of the cell cycle is governed by the D-type cyclins (D1, D2, D3) (1, 2). These proteins are induced at the beginning of the G1 phase and associate with serine/threonine cyclin-dependent kinases (Cdk) to form holoenzymes (3 - 5). Overexpression of cyclin D1 in human tumors as well as in several cancer cell lines has been reported (6). Cyclin D/Cdk complexes are specific for the cell type (3 - 5). An important substrate for these kinases is the product of the retinoblastoma susceptibility gene (RB1). In G1 phase underphosphorylated RB1 binds E2F transcription factors and prevents the cell cycle progression. During mid-to-late G1, RB1 is phosphorylated, releases E2F-dependent transcription and the cells advance to S-phase (7). The fact that cyclin D-dependent kinase activity is only required for cell cycle progression in cells expressing functional RB1 (8), is in agreement with the essential role of RB1 as a substrate of cyclin D-dependent kinases.

Sodium butyrate is an inducer of cell differentiation and inhibitor of proliferation in numerous cell lines, acting probably via the inhibition of protein desacetylases and the resulting selective changes in gene expression (9 - 11). The genes affected by this agent are on the one hand those characteristic for the differentiated phenotype of the particular cell line (e.g. 12 - 14), and on the other hand those coding for proteins involved in the regulation of cell cycle progression such as c-myc (15), cdc2 (16) or $p21^{Waf\text{-}1}$ (17). DNA sequences conferring the induction of indicator gene expression by sodium butyrate (butyrate-response element; BRE) under transient transfection conditions were identified in the 5′-flanking regions of several genes, including calbindin 28 kDa (11).

Incubation in a sodium butyrate-containing medium generally leads to the accumulation of cells in the G1 phase. We have shown recently that sodium butyrate abolishes RB1 phosphorylation in the chemically transformed mouse fibroblasts BP-A31 during serum-induced G1 progression, and suggested a possible role of $p21^{Waf\text{-}1}$ (18). In this work, we have studied the regulation of cyclin D1 gene expression. Our data indicate that sodium butyrate inhibits cyclin

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D1 promoter activity, probably by modifying the activity of factors interacting with a sequence homologous to BRE located in the promoter region of the cyclin D1 gene.

MATERIALS AND METHODS

Cell lines, culture and synchronization. The chemically transformed mouse fibroblasts BP-A31 were maintained in the MEMα medium supplemented with 6% fetal bovine serum (FBS). To synchronize these cells in early G1 phase, they were placed for 1 to 2 days in serum-free MEMα with added 2.5 μM $FeSO_4$. The human cancer cell lines KB-3-1 (epidermoid) and MDA-MB-231 (breast) were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% FBS.

Western blot analysis. Cell extracts were prepared as described earlier (18, 19) and electrophoresed in a 8% SDS-PAGE (80 μg protein per lane). The proteins were transferred onto a nylon membrane and revealed by incubation with 0.5 μg/ml of rabbit polyclonal antibodies C-15 (anti-RB1) or anti-cyclin D1 (Santa Cruz) using the enhanced chemoluminiscence detection system (Amersham).

RNA analysis. Total cellular RNA was extracted by the LiCl/urea method and analyzed by Northern blotting with the cyclin D1 cDNA (20) and α-tubulin cDNA (21).

Recombinant plasmids. BREcal28CAT (containing the BRE sequence of the calbindin 28 kDa promoter) and BREcycD1CAT (containing the homologous 11 base pair sequence situated between -343 and -333 bp in the human cyclin D1 promoter) were constructed by inserting double stranded oligonucleotides TAGGCCT**AGCAAGCTCCA**AG (BREcal28) or TAGGCCT**AGCCACCTCCA**CG (BREcycD1CAT; nucleotides within the consensus are in bold print) into the HindIII/BamHI site of the pBLCat2 expression vector (22), upstream of the complete herpes virus thymidine kinase promoter.

Transient transfection assay. Exponentially growing BP-A31 cells in 10 cm dishes (10^6 cells/dish) were transfected by the calcium phosphate method with 3 μg of D1Δ-973 (23), BREcal28CAT, or BREcycD1CAT and 7 μg of carrier (Bluescript). After 16h, the cells were placed in a fresh medium, either complete or serum-free (24 h), and incubated with sodium butyrate (6mM) as described in Results. The cells were harvested and assayed for luciferase or chloramphenicol acetyl transferase activity (CAT). We have verified that the variations in the protein concentration of the cell extracts as a function of treatment were insignificant. We preferred to relate the indicator gene product activities to the concentration of cellular proteins rather than to the activity of a cotransfected internal standard plasmid as viral promoters (used in such plasmids) turned out to be sodium butyrate-induced (cf. 24).

RESULTS

Effect of Sodium Butyrate on the Cell Contents of Cyclin D1 Protein and mRNA

In an earlier study (25), we have noted that cell proliferation was inhibited by sodium butyrate in a concentration-dependent manner. While a 3 mM concentration of the drug was sufficient to cause a strong inhibition, 6 mM sodium butyrate was required for a total growth arrest. Incubation with 3 mM sodium butyrate did not lead to a change in the state of phosphorylation of RB1 in the human epidermoid cancer cell line KB-3-1, whereas 6 mM butyrate caused a time-dependent RB1 dephosphorylation. In the following experiments, we have chosen the higher sodium butyrate concentration, with the aim of completely blocking the cell cycle.

In the serum-deprived, quiescent BP-A31 cells, cyclin D1 was strongly expressed (Fig. 1, lane 1). Sodium butyrate caused a time-dependent decrease in the contents of cyclin D1 (lanes 2 and 3). Incubation of quiescent BP-A31 cells with serum led to a further increase in the level of cyclin D1, concommittantly with the hyperphosphorylation of RB1. This effect of serum was partially inhibited by butyrate (lanes 4 and 5 *vs.* 6 and 7); concommittantly, butyrate prevented the hyperphosphorylation of RB1, in agreement with our earlier data (18). Under the conditions of exponential growth in a serum-containing medium, butyrate also caused a reduction of the cell content of cyclin D1 (data not shown).

The decrease in the cell content of cyclin D1 protein in quiescent BP-A31 cells incubated with sodium butyrate was paralleled by the decreased levels of the cyclin D1 mRNA (Fig. 2). This effect of butyrate was also observed in the presence of cycloheximide (compare lanes 5 and 6), indicating that it did not require continuing protein synthesis.

We have also studied the effect of butyrate on cyclin D1 expression in the human cancer cell lines MDA-MB-231 and KB-3-1 under the conditions of exponential growth (Fig. 3). In

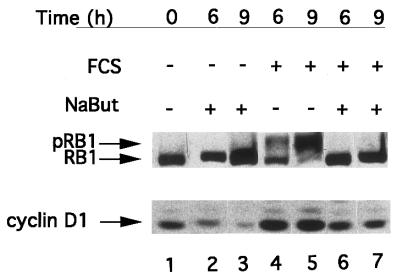

FIG. 1. Effect of sodium butyrate on RB1 phosphorylation state and on the cell content of cyclin D1 protein in BP-A31 cells. Serum-deprived BP-A31 cells (lane 1) were incubated for different time periods with sodium butyrate (6mM) (lanes 2 and 3) with 6% FCS (lanes 4 and 5), or both (lanes 6 and 7). The cell extracts were analysed by Western blotting with antibodies against RB1 and cyclin D1. pRB1 represents the hyper-phosphorylated form, RB1 the fast-migrating, hypophosphorylated form of the RB1 gene product.

the former, there was no detectable change in the cyclin D1 signal on western blot, whereas in the latter, cyclin D1 level rapidly decreased upon incubation with this compound. As in the case of quiescent BP-A31 cells, sodium butyrate caused a rapid decrease of cyclin D1 mRNA in the KB-3-1 cells, by a mechanism independent of protein synthesis (data not shown).

Sodium Butyrate Inhibits the Activity of Cyclin D1 Promoter

In order to study the mechanism by which sodium butyrate modifies the cellular level of cyclin D1, we have used a construct in which an indicator gene (firefly luciferase) was placed downstream of the promoter region of the human cyclin D1 gene. This construct was transfected into BP-A31 cells which were then incubated for 24h in a serum-free medium in order to be made quiescent prior to incubation with sodium butyrate. As shown in Fig. 4A, the expression of the indicator gene was inhibited by butyrate. The differences between the enzyme activities in extracts of cells incubated without and with butyrate were

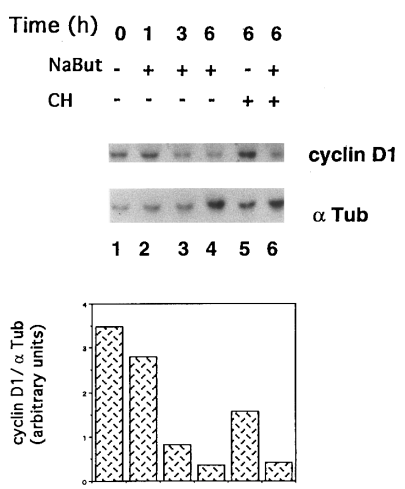

FIG. 2. Effect of sodium butyrate on cyclin D1 mRNA contents in quiescent BP-A31 cells. Serum-deprived BP-A31 cells (lane 1) were incubated for different time periods with sodium butyrate (6 mM) (lanes 2, 3 and 4), with cycloheximide (1μg/ml; lane 5) or both (lane 6). Twenty μg of total RNA were analysed by Northern blotting for cyclin D1 and α-tubulin (α-Tub) mRNA. Upper panel: autoradiography; lower panel: ratio between the cyclin D1 mRNA and the α-tubulin mRNA evaluated by densitometry (arbitrary units).

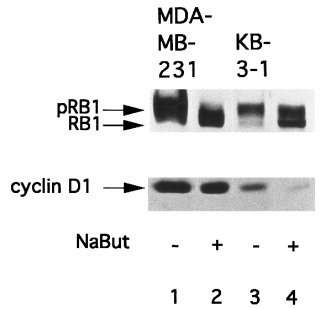

FIG. 3. Effect of sodium butyrate on cyclin D1 and RB1 proteins in the KB-3-1 and MDA-MB-231 cells. Exponentially growing cells were cultured for 24h without (−) or with (+) sodium butyrate (6 mM). Cell lysates were analyzed by Western blotting with anti-cyclin D1 and anti-RB1 antibodies as in Fig. 1.

observed after a delay of >4h, and after 9h, luciferase activity was more than 5 times lower in cells incubated with butyrate than in the controls. In another experiment, we have observed a decreased expression of the indicator gene from 6h onwards (data not shown). It is to be noted that under these experimental conditions, the effect of butyrate is in fact underestimated as the accumulation of the indicator gene transcript is undisturbed during the time between transfection and the addition of the drug.

Inhibition of cyclin D1-promoter activity by butyrate was also observed in exponentially growing BP-A31 cells (Fig. 4B).

In the promoter region of the cyclin D1 gene (23), a sequence of 11 nucleotides situated between −343 and −333 bp upstream of the transcription initiation site displays a significant homology to the calbindin 28K BRE sequence (11). Similar sequences are present in the HIV and MoMuSV long terminal repeats, as well as in the promoter of the metallothioneine II gene, and notably in the regions described to confer inducibility of transcription by sodium butyrate (26 - 28). Consequently, we have considered the possibility that this 11bp element of the cyclin D1 promoter might mediate the inhibition of transcription. In fact, enhancer or silencer activity of DNA elements depends on their sequence as well as on local context (see 29 for a review). Transient transfection experiments carried out with the BP-A31 cells brought about evidence in support of this hypothesis (Fig. 5). The calbindin 28 kDa BRE element (BRE^{cal28}) conferred an approximately 2-fold induction of the indicator gene (CAT) expression by 4 to 6 mM sodium butyrate. In contrast, the expression of CAT was decreased approximately 3-fold by butyrate when the ''BRE-like'' element from the cyclin D1 promoter (''BRE^{cycD1}'') replaced the calbindin 28K BRE sequence. The butyrate-induced differences in the indicator gene expression were observed in several independent experiments.

DISCUSSION

Sodium butyrate is a natural molecule, produced by bacteria and present in high concentrations in the human colon where it serves as a source of energy and supposedly protects the colonic epithelium from carcinogenic effects of toxic products present in the fæces. This compound is known since a long time as an inducer of growth arrest and differentiation but the mechanisms involved have not been elucidated. The effects of sodium butyrate are presumably due to nonspecific but highly selective alterations in gene expression. It is intriguing that sodium butyrate inhibits the cell cycle progression in virtually all cell types. This fact may be the consequence of changes in the expression of a small number of genes coding for proteins essential for the cell cycle. The identification of the cellular targets of sodium butyrate can be therefore important for the understanding of the mechanisms of regulation of the proliferation of normal and cancer cells.

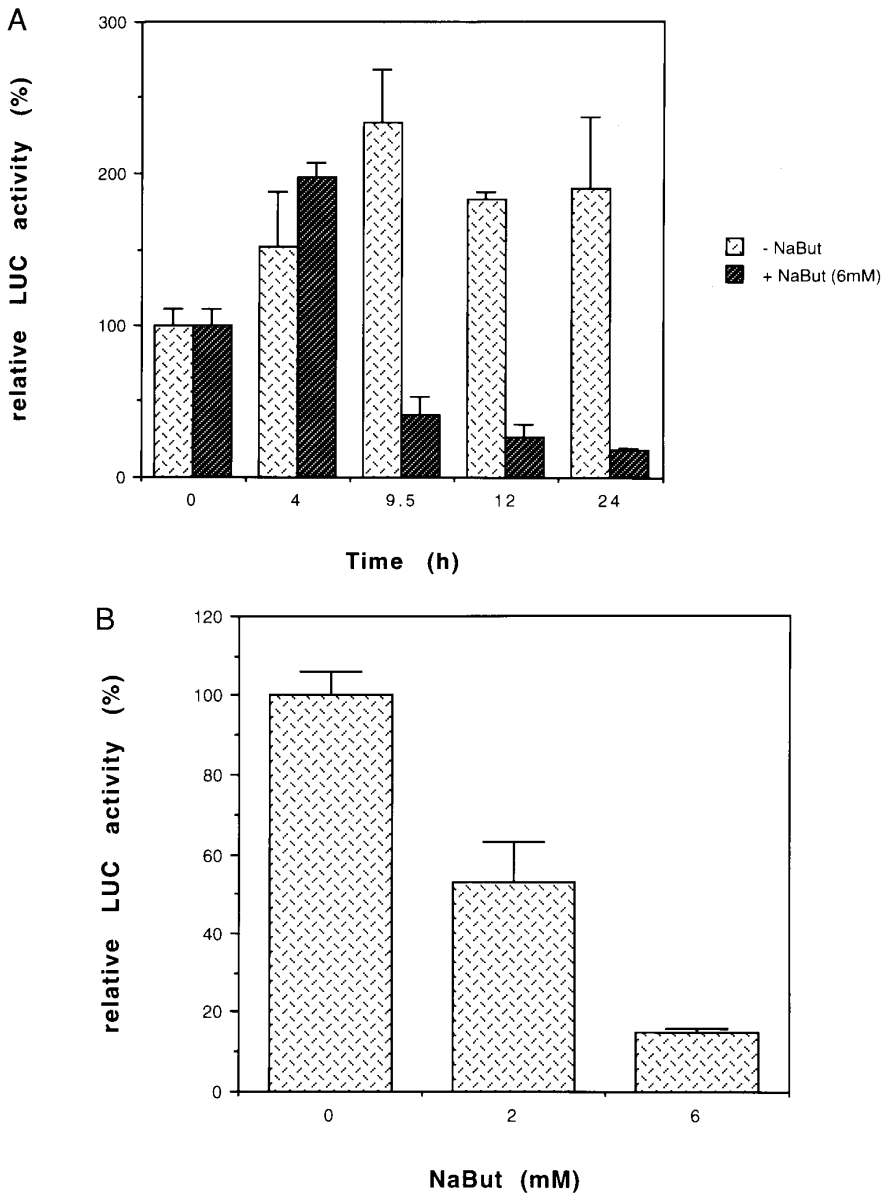

FIG. 4. Effect of sodium butyrate on the activity of cyclin D1 promoter. BP-A31 cells were transfected with 3 μg of the D1Δ-973 plasmid plus 7μg of carrier DNA (Bluescript) and allowed to recover during 16h. A. The cells were serum-deprived during 24h prior to the addition of sodium butyrate (t = 0), and then harvested at the times indicated. B. The cells were placed for 24h in fresh (complete) medium containing sodium butyrate at 0, 2 mM or 6 mM concentrations, and then harvested. Luciferase activity was determined in cell lysates and normalized for protein concentration. The results shown are means $\pm$ range of duplicates.

Our results suggest that sodium butyrate inhibits of cyclin D1 gene expression on transcriptional level, and that the BRE-like element situated in the 5′-region of this gene may be implicated. To our knowledge, this is the first example of transcription-inhibitory activity of butyrate demonstrated under transient transfection conditions. The effect of butyrate is not due to an interference with the cell cycle progression as the experiments were done with G0/G1

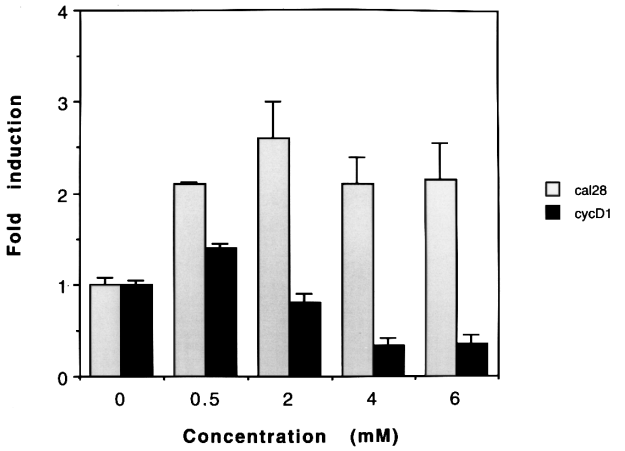

FIG. 5. Effect of sodium butyrate on the expression of BRE^{cal28} CAT and ''BRE^{cycD1}''CAT in transiently transfected BP-A31 cells. Exponentially growing BP-A31 cells were transfected with 3 μg of BRE^{cal28} CAT (cal28) or ''BRE^{cycD1}''CAT (cycD1) plasmid plus 7 μg of Bluescript. The cells were maintained during 16 hours with the appropriate Ca-phosphate precipitated plasmid DNA and subsequently placed for 24 hours in a fresh medium containing sodium butyrate as indicated. CAT activity was determined in the cell lysates and normalized for protein concentration. The results shown are means ± ranges of duplicates.

synchronized cells. The expression of cyclin D1 in the quiescent BP-A31 cells is probably due to the fact that in these cells, the p21ras protein is present as a complex with GTP (i.e. in its active form) even in the absence of exogenous growth factors (30).

The inhibition of the cyclin D1 promoter activity was observed only after a delay of more than 4h, whereas the decrease in the cell contents of mRNA was already significant after 3h of incubation of the cells with sodium butyrate. This discrepancy may reflect a difference between the effect of the drug on the transcription of a transiently transfected (episomal) gene and a chromatin gene. Alternatively, it is possible that butyrate may accelerate the degradation of cyclin D1 mRNA, in addition to its effect on gene transcription.

In spite of the fact that sodium butyrate inhibits both cyclin D1 gene expression and RB1 hyperphosphorylation, these two effects are probably not causally linked. In fact, the inhibition of RB1 hyperphosphorylation by butyrate requires transcription (18) whereas the inhibition of cyclin D1 expression appears to be direct. Moreover, certain molecular mechanisms of butyrate action are different according to cell line: thus, in the MDA-MB-231 cells, butyrate did not affect cyclin D1 level, although the proliferation of these cells is efficiently blocked by the compound, and RB1 phosphorylation is inhibited (Lallemand, unpublished data). Induction of $p21^{Waf-1}$ and possibly of other growth inhibitory proteins by sodium butyrate are likely to play a role in its antiproliferative activity.

The modifications of gene expression by sodium butyrate are probably due to the inhibition of histone deacetylases. In this context, it is interesting that a key protein involved in the regulation of the activity of several transcription factors, P/CAF, displays histone acetylase activity (31); complexes of P/CAF with p300/CBP inhibit the exit from G0/G1. We propose that the (non-targeted) hyperacetylation of histones caused by sodium butyrate may mimic the targeted acetylation of histones by P/CAF; thus, sodium butyrate would reproduce the cell cycle inhibitory effect of P/CAF.

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