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Transcriptional Regulation of Genes Encoding Insulin, Glucagon, and Angiotensinogen by Sodium Butyrate in a Rat Islet Cell Line

JACQUES PHILIPPE,[1] DANIEL J. DRUCKER,[1] WILLIAM L. CHICK,[2] AND JOEL F. HABENER[1,3]*

Laboratory of Molecular Endocrinology, Massachusetts General Hospital,[1] and Howard Hughes Medical Institute, Harvard Medical School,[3] Boston, Massachusetts 02114, and Departments of Biochemistry and Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605[2]

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The state of differentiation of various neoplastic cell lines is inversely correlated with the rate of cellular growth. To delineate the changes in hormone gene expression associated with an induced decrease in the growth rate of rat insulinoma cells, we studied the effects of sodium butyrate on the expression of the genes encoding insulin, glucagon, and angiotensinogen. Sodium butyrate inhibited cellular proliferation and decreased levels of c-*myc* mRNA. Concomitantly, steady-state levels of mRNAs encoding insulin and glucagon increased by 10- and 8.5-fold, respectively, as a result of a specific increase in the transcription of both genes. Sodium butyrate also inhibited angiotensinogen gene expression, which was ectopic in the insulinoma cells. These observations suggest that sodium butyrate induces a pattern of events leading to the differentiation of the rat insulinoma cells.

The mechanisms involved in cellular differentiation and associated changes in the expression of specific genes have received considerable attention. Various neoplastic cell lines show characteristic changes of differentiation after treatment with specific chemical agents. The Friend erythroleukemia cell line differentiates in response to sodium butyrate (20, 21), whereas the promyelocytic leukemia cell line, HL-60 (6), and the medullary thyroid carcinoma cell line differentiate during exposure to vitamin D and tumor-promoting agents (8), respectively. Sodium butyrate, a short-chain carboxylic acid, induces a variety of morphological and physiological changes in different cell types, including reversible changes in cellular morphology and growth rate (14) and inhibition of DNA synthesis and cell division (16). Moreover, sodium butyrate specifically stimulates the synthesis of a number of cellular proteins (for a review, see reference 27), as well as transcription of the metallothionein (1) and chorionic gonadotropin (13) genes. These effects on protein synthesis and gene transcription correlate with changes in chromatin structure in association with hyperacetylation of histones, particularly H_3 and H_4, and of high-mobility group proteins (HMG-14 and HMG-17) (29, 31). Histone hyperacetylation does not appear, however, to be a prerequisite for cell differentiation (12, 30). Other responses of sodium butyrate have been observed with regard to the hypomethylation of DNA (5, 9), the altered sensitivity of bulk chromatin and specific genes to nuclear digestion (10, 32), and the selective inhibition of histone H-1 and H-2 phosphorylation (2).

Previously, we characterized several clonal islet cell lines that express multiple hormone genes, including insulin, glucagon, somatostatin, and angiotensinogen (J. Philippe, W. L. Chick, and J. F. Habener, J. Clin. Invest., in press). These rat insulinoma (RIN) cells are neoplastic cells derived from an X-ray-induced rat pancreatic islet tumor (4), which express some of the differentiated functions of normal islet cells, including the biosynthesis and secretion of peptide hormones and the expression of L-dopa decarboxylase (26). The cells are capable of continuous growth and are multipotential in their expression of different polypeptide hormone genes (Philippe et al., J. Clin. Invest., in press). To investigate the effects of islet cell differentiation on peptide hormone gene expression, we studied the effects of sodium butyrate on the growth and expression of hormone-encoding genes in the RIN cells.

RIN 1056 cells, isolated from an X-ray-induced pancreatic islet cell tumor, express the insulin, glucagon, somatostatin, and angiotensinogen genes (11; A. R. Brasier, J. Phillipe, D. J. Campbell, and J. F. Habener, J. Biol. Chem., in press; Philippe et al., J. Clin. Invest., in press). Cells were grown in Dulbecco modified Eagle medium at a glucose concentration of 4,500 mg/liter. Sodium butyrate (1 to 2 mM) induced changes in both the morphology and growth rate of the RIN cells. Within 24 h, the size of the cells increased owing to

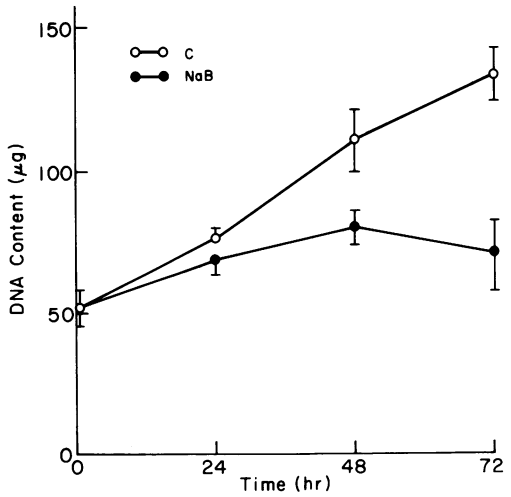

FIG. 1. Effect of sodium butyrate on DNA synthesis. RIN cells (10^7 cells per dish) were incubated in the presence (NaB) or absence (C) of sodium butyrate (2 mM) for 24, 48, and 72 h. The DNA content was measured by fluorometry (19). Data show means ± standard deviations of three experiments.

* Corresponding author.

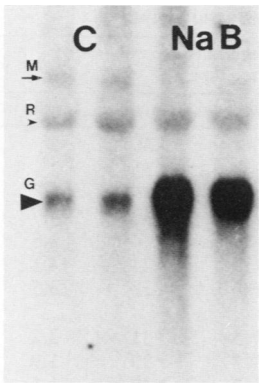

FIG. 2. Effect of sodium butyrate on c-*myc* mRNA levels. RIN cells were incubated for 72 h in the absence (C) or presence of sodium butyrate (NaB) (2 mM). Total RNA was then extracted, size fractionated on an agarose gel, and electroblotted to a nylon membrane (see text). The blot was hybridized with ^{32}P-labeled cDNA probes complementary to the c-*myc* and glucagon mRNAs. Symbols: M, c-*myc* mRNAs; G, glucagon mRNA; R, 18S rRNA.

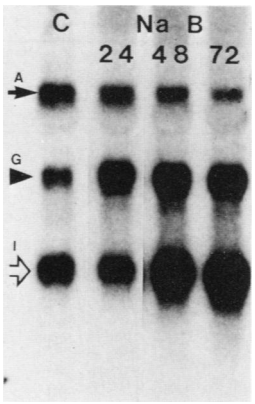

FIG. 3. Sodium butyrate-increased glucagon and insulin mRNA levels. Northern blot analysis of total RNA extracted from control and sodium butyrate-treated cells. Cells were incubated with or without 2 mM sodium butyrate for 24, 48, and 72 h. The blot was hybridized with ^{32}P-labeled cDNA probes complementary to angiotensinogen, glucagon, and insulin mRNAs. Symbols: A, angiotensinogen mRNA; G, glucagon mRNA; I, insulin mRNA.

enlargement of both the nucleus and cytoplasm. These changes stabilized between 48 and 72 h and were reversible. Sodium butyrate both inhibited the growth of the cells (DNA content as measured by fluorometry [19] was decreased to 55% of the control value after 3 days of treatment; Fig. 1) and decreased cellular levels of c-*myc* mRNA (Fig. 2).

We next determined by Northern blot analyses (J. Philippe, D. Drucker, and J. F. Habener, J. Biol. Chem., in press) the effects of sodium butyrate on the expression of the genes encoding insulin and glucagon, which are normally expressed in the pancreatic islets (J. Philippe, J. Clin. Invest., in press) and angiotensinogen, which is ectopically expressed in the RIN cells (Brasier, in press) (Fig. 3 and 4). Synthetic oligonucleotides from 26 to 43 bases, complementary to different regions of each of the mRNAs (six for rat glucagon, six for rat angiotensinogen, four for rat insulin, two for rat actin, and one for rat c-*myc*), were labeled at the 5′ end with [^{32}P]ATP by T4 polynucleotide kinase and used as hybridization probes.

Glucagon mRNA levels had increased at 6 h after addition of sodium butyrate and reached maximum levels (8.5-fold increase over control levels) at 48 h, with little additional increase between 48 and 72 h. The kinetics of the accumulation of insulin mRNA were different from those of glucagon mRNA, inasmuch as at 24 h, the increase was smaller and progressively linear up to at least 72 h, reaching 10-fold over control levels. By contrast, angiotensinogen mRNA levels decreased to 32% of the control levels by 72 h.

To further investigate the mechanisms of action of sodium butyrate on gene expression, we measured nascent insulin, glucagon, and β-actin (as a control) RNA transcripts in both control and sodium butyrate-treated cells by a nuclear run-on transcription assay (22, 23). We found that the transcription of both the insulin and glucagon genes was increased by sodium butyrate and that the increases were apparent within 6 and 12 h of treatment, respectively. Glucagon and insulin gene transcription increased by 9.3- and 5.3-fold, respectively (Table 1).

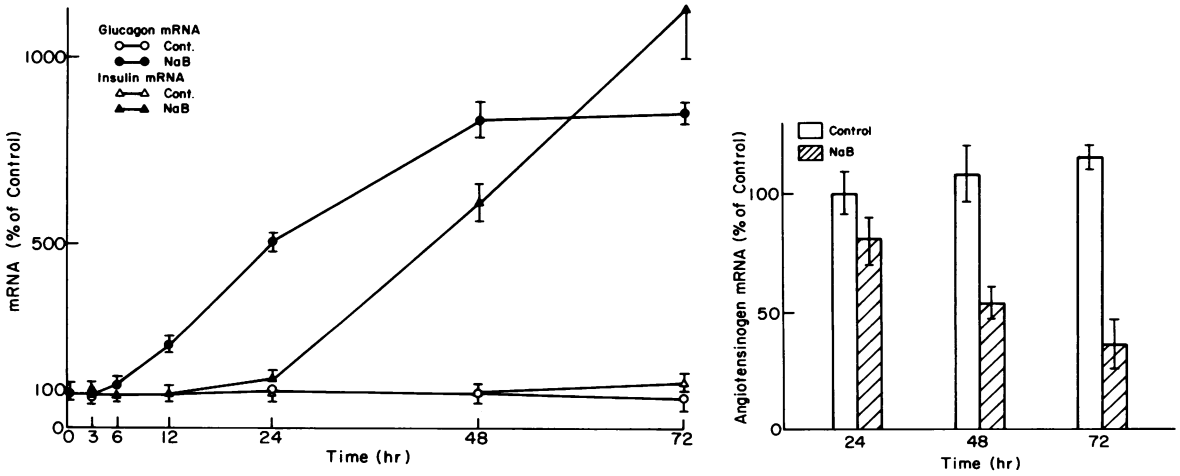

FIG. 4. Time course of glucagon and insulin (left) and of angiotensinogen (right) mRNA accumulation after treatment with sodium butyrate (2 mM). Cells were incubated in the presence (NaB) or absence (Cont.) of sodium butyrate for 3, 6, 12, 24, 48, and 72 h. Quantification of each mRNA was done by densitometric scanning of the autoradiograms. Values represent means ± standard deviations of five experiments, and glucagon-, insulin-, and angiotensinogen-specific mRNAs were normalized from the internal control of β-actin mRNA.

TABLE 1. Effect of sodium butyrate on insulin and glucagon gene transcription[a]

Expt	Time (h)	Input of ^{32}P-labeled RNA (cpm, 10^6)[b]	^{32}P bound to cDNA immobilized on filters (cpm)				^{32}P specifically hybridized (cpm)			Gene transcription (ppm)[c]		
			Insulin	Glucagon	PBR322	Actin	Insulin	Glucagon	Actin	Insulin	Glucagon	Actin
1	C[d]	3.9	48	44	40	76	8	4	36	5	2	23
	C	3.9	49	48	46	77	3	2	31	2	1.3	20
	1	3.9	42	41	36	72	6	5	36	3.8	3.2	23
	6	2.7	48	56	44	76	4	12	32	3.7	11.1	29
	6	2.7	51	64	48	92	3	16	44	2.8	14.8	40
	12	2.3	54	58	41	65	13	17	24	14.1	18.5	26.7
	12	2.3	72	75	55	82	17	20	27	18.5	21.7	25.3
2	C	5	60	61	56	87	4	5	31	2.2	2.5	16
	1	3.9	44	41	43	70	3	2	29	1.9	1.3	18.6
	6	3.1	30	40	28	54	2	12	26	1.6	10	21
	6	3.1	34	41	28	54	6	13	26	4.8	10.5	21
	12	2.3	47	44	32	52	15	12	20	16.3	13	21.7

[a] Confluent plates of RIN cells were incubated with or without sodium butyrate (2 mM) for 1, 6, and 12 h. Nuclear run-on transcription assays were performed. Nuclei were isolated (22), and the equivalent of 400 μg of DNA was incubated in the presence of 1 mM ATP, GTP, and CTP and 500 μCi of [α-^{32}P]UTP for 30 min at 26°C (23). Nuclear RNA transcripts were hybridized to an excess (5 μg) of rat glucagon (17) and rat insulin (provided by H. M. Goodman, Harvard University) and actin cDNAs (provided by D. Cleveland, The John Hopkins University School of Medicine) and immobilized on nitrocellulose filters for 48 h. Counts for individual filters were measured for 60 to 90 min. Machine background was 16 to 20 cpm. Hybridization efficiency was controlled through the addition of [^{3}H]UTP-labeled glucagon (17) and insulin cRNAs to either hybridization solution or to parallel hybridization reactions. Control nuclei were isolated 6 h (experiment 1) and 12 h (experiment 2) after the addition of fresh medium.

[b] In hybridization reaction.

[c] Corrected for hybridization efficiency.

[d] C, Control nuclei.

Although the increase in gene transcription corresponded to the maximal increase in accumulation of insulin and glucagon mRNA levels observed after treatment with sodium butyrate, we also evaluated the effects of sodium butyrate on the stability of both of these mRNAs. To inhibit RNA synthesis (7) and to measure the decay rate of total RNA and specific mRNAs, we added actinomycin D (5 μg/ml) to the cells and extracted RNA after 6, 12, and 24 h. Changes in total RNA were measured by spectrophotometry, and specific mRNAs were measured by Northern blot analysis. Sodium butyrate did not alter the decay rates of total RNA, glucagon mRNA, or insulin mRNA. The calculated half-lives of glucagon and insulin mRNAs were 12 and 18 h, respectively (data not shown).

Concentrations of immunoreactive insulin and glucagon were measured in the culture medium by radioimmunoassays (11, 24) to determine whether the increase in mRNA levels was reflected at the levels of hormone biosynthesis. Amounts of insulin and glucagon increased in the culture medium nine- and fivefold, respectively (data not shown). No increase in either hormone was detected within the first 6 h of incubation with sodium butyrate, an indication that the increased amounts of the hormone detected in the medium probably reflect new hormone biosynthesis rather than acute release of preformed hormone.

The results of these studies indicate that sodium butyrate induces certain differentiated features in the RIN cells. Although retaining some of the features of the normal islets (for example, peptide hormone biosynthesis and secretion), these neoplastic islet cells display several undifferentiated characteristics: (i) they grow as an immortalized line, (ii) levels of peptide hormone gene expression (for example, insulin mRNA levels) are estimated to be 1/200 of those in normal islets, (iii) the cells demonstrate multipotentiality in expressing these genes (26; Philippe et al., J. Clin. Invest., in press), (iv) they lack responsivity of insulin secretion to glucose, and (v) the angiotensinogen gene is ectopically expressed. Treatment of the RIN cells with sodium butyrate resulted in decreases in DNA synthesis and levels of c-*myc* and angiotensinogen mRNAs and increases in the transcription of the glucagon and insulin genes. These data, along with the observation that somatostatin gene expression is also enhanced by sodium butyrate in a similar RIN cell line (15), suggests that these cells acquire more differentiated characteristics.

The kinetics of the increases in the insulin and glucagon mRNA levels differ in response to sodium butyrate. Although sodium butyrate increases the amounts of both insulin and glucagon mRNAs to approximately the same extent, the induction of insulin gene transcription is delayed compared with that of the glucagon gene. In addition, the half-life of the insulin mRNA is about twice as long as that of the glucagon mRNA. The combination of the delayed increase in insulin gene transcription and long half-life of the insulin mRNA explains not only the small increase in insulin steady-state mRNA levels observed at 24 h but also the continued accumulation of the insulin mRNA at a time when glucagon mRNA levels have reached a plateau. The decrease in c-*myc* expression corresponds to the decrease in DNA synthesis, and c-*myc* gene regulation is known to correlate with cell proliferation in various systems (18). Cellular proliferation arrested in the G_0 phase of the cell cycle is associated with a decrease in c-*myc* expression in the human promyelocytic leukemia (HL-60) (28), the murine teratocarcinoma stem (3), and the human medullary thyroid carcinoma cell lines (8).

Our findings of an inverse relationship between the state of proliferation of the RIN cells and expression of the insulin and glucagon genes are similar to the six- to eightfold induction of insulin mRNA levels in RIN 5F cells, which enter a transient state of arrested growth when cultured in serum-free, hormonally defined medium on an extract of extracellular matrix derived from a basement membrane-secreting tumor line (25). In addition, these cells, normally unresponsive to glucose, increased insulin secretion by threefold in the presence of high glucose. These observations further illustrate the link between the state of growth and the acquisition of more differentiated features.

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