Vol. 193, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 28, 1993 Pages 406-412

# INDUCTION OF RECOMBINANT HUMAN γ-GLUTAMYL TRANSFERASE BY SODIUM BUTYRATE IN TRANSFECTED V79 AND CHO CHINESE HAMSTER CELLS

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Received March 30, 1993

SUMMARY Sodium butyrate was used to enhance biosynthesis rates of recombinant human γ-glutamyl transferase (GGT) expressed under the control of the SV40 or the cytomegalovirus immediate early promoter, respectively, in transfected V79 and CHO Chinese hamster cell lines. Maximal induction of GGT specific activity in butyrate-treated cells ranged from 3 to 5-fold and resulted from a strong increase in the GGT mRNA ratio. We also observed that maximal transcription level in V79 cells occured within 12 hr of treatment, whilst the cell proliferation was transiently arrested. Despite its processing requirements, induced GGT exhibited unchanged catalytic and physico-chemical features relative to human serum or hepatoma enzyme, thus appearing as an excellent model for further studies on human GGT. © 1993 Academic Press, Inc.

INTRODUCTION γ-Glutamyl transferase (GGT, EC 2.3.2.2) is a plasma membrane-anchored extracellular enzyme, synthesized as a precursor which is further glycosylated and cleaved into an heterodimer (1). Although GGT is known to be involved in several biochemical pathways, such as glutathione catabolism, mercapturic acid biosynthesis or leukotrienes metabolism (2, 3, 4), its physiopathological significance has not yet been clearly elucidated.

In view of structural and functional studies, we established different transfected cell lines stably expressing high levels of recombinant human GGT, which exhibit similar properties as the native enzyme (5, 6). In order to ease the purification process as well as to obtain larger amounts of pure enzyme for further analyses, we focused our attention on increasing the productivity of our expression systems. A number of stimulating agents are known for their potency in enhancing protein biosynthesis in cell lines; amongst them is sodium butyrate, which can induce reversible changes in the cells (7), especially an increased expression of recombinant proteins (8, 9).

This paper demonstrates the enhanced synthesis of recombinant human GGT in genetically engineered Chinese hamster lung - V79 - and ovary - CHO - cells in response to treatment with sodium butyrate. It also provides molecular information on the induction mechanism and the properties of the induced enzyme.

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[c] *Equal contribution to this work.*

ABBREVIATIONS: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylene diamine tetraacetic acid; FCS, fetal calf serum; GGT, γ-glutamyl transferase; GluCNA, L-γ-glutamyl-3-carboxy-4-nitroanilide; HPLC, high performance liquid chromatography; NaBu, sodium butyrate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

0006-291X/93 $4.00
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## MATERIALS AND METHODS

Media and chemicals. Dulbecco's modified Eagle's medium (DMEM), DMEM/Ham F12 (1/1), the 1X trypsin-EDTA and the antibiotic-antimycotic solutions were from GIBCO. Fetal calf serum (FCS) was from Boehringer Mannheim, sodium butyrate (NaBu) from Merck. [$\alpha$-$^{32}$P] dCTP and Hybond C nitrocellulose membrane were from Amersham, whereas Immobilon-P membrane was from Millipore. The other chemicals were of the highest grade available.

Cell lines and culture conditions. The GGT-producing cell lines, namely V79HGGT and CHO-GGT, were obtained as described earlier, after introducing the cDNA coding for the human hepatoma Hep G2 GGT (10) under the control of the SV40 or the cytomegalovirus promoter, respectively (5, 6). Both transfected cell lines expressed recombinant GGT activity to an average level of 2 U/mg protein.

All cells were cultured at 37°C in a humidified 5% $CO_2$/95% air atmosphere. V79 cells were grown in DMEM, supplemented with 5% (v/v) heat-inactivated FCS and 1X (final concentration) antibiotic-antimycotic solution. CHO cells were cultured in DMEM/Ham F12 medium, supplemented with 4 mM L-glutamine and 5% (v/v) heat-inactivated FCS. At the end of the culture, viable cells were either scraped and kept at -20°C as dry pellets until enzyme and protein analyses were begun, or treated with 0.05% (v/v) trypsin for cell numeration.

Induction experiments. NaBu was prepared as a 1-M stock solution, pH 7.0, sterilized by ultrafiltration and conserved at -20°C until use. Cells were seeded in 100-mm diameter Petri dishes at the density of 1 or $2 \cdot 10^6$ cells, and grown for 48 hr under standard culture conditions. Induction was initiated by substituting fresh complete medium supplemented with various concentrations of NaBu (0.5 to 10 mM). When treatments were applied for 48 hr, the medium was renewed after 36 hr. Each determination needed 6 separate dishes: 3 for counting the living cells, 3 for measuring specific GGT activity.

GGT assays. Cell pellets were resuspended in phosphate-buffered saline. Standard conditions were as described earlier (11), with 6 mM L-$\gamma$-glutamyl-3-carboxy-4-nitroanilide (GluCNA) and 150 mM glycylglycine (Gly-Gly) as donor and acceptor substrate concentrations. The enzymatic unit (U) was defined in (5). $K_m$ were determined for both GluCNA and Gly-Gly as described (11).

RNA analysis. Total RNA was prepared from confluent cells by the guanidium thiocyanate extraction method according to Chomczynski and Sacchi (12) and concentrations were estimated by absorbance measurements at 260 nm. Slot blot analysis was performed using the Bio-Dot Slot apparatus (Bio-Rad) for adsorbing known amounts of RNA on a Hybond C nitrocellulose membrane. Samples were hybridized overnight at 42°C to the total human hepatoma Hep G2 GGT cDNA (10) labelled by random priming with [$\alpha$-$^{32}$P] dCTP (3000 Ci/mmol; 1 Ci = 37 GBq). Filters were washed under stringent conditions as previously described (5) and GGT mRNAs were detected by autoradiography.

Protein analyses. Protein concentrations were estimated according to Lowry *et al.* (13), using bovine serum albumin as standard. Protein content in the cell samples was analyzed after SDS-PAGE, essentially as described by Laemmli (14). Proteins were then transferred to Immobilon-P membranes using a semi-dry transfer apparatus and GGT polypeptides were specifically detected with an antiserum raised against GGT from human kidney (5).

## RESULTS

Effects of NaBu on GGT specific activity and cell growth. The inducer potency of NaBu added to the culture medium was investigated with the view of increasing the biosynthesis of active recombinant human GGT in our GGT-producing cell lines. We first made sure that no endogenous GGT activity appeared in parental V79 and CHO cell lines after treatment with NaBu. On the contrary, when the transfected cell lines were treated, an important increase of specific GGT activity proportional to the NaBu concentration ($\leq$ 5 mM) in the medium was observed after 24 hr (Fig. 1a). Induction of GGT activity, though evolving similarly in either cell line, was found to be higher in V79HGGT cells than in CHO-GGT cells when treated with 10 mM NaBu, with a 480% and a 320% enhancement, respectively.

The effects of NaBu were also examined throughout the treatment. As shown on Fig. 1b, GGT activity in V79HGGT cells seemed to reach a maximal value within 24 hr and did not increase any more if the treatment was prolonged. As depicted by Fig. 1b, renewal of the medium at 36 hr gave no effect on GGT activity. These data indicate that the limitation of induction did not result from an alteration of the medium. Moreover, NaBu was apparently not degraded or utilized by the cells throughout the culture, as confirmed

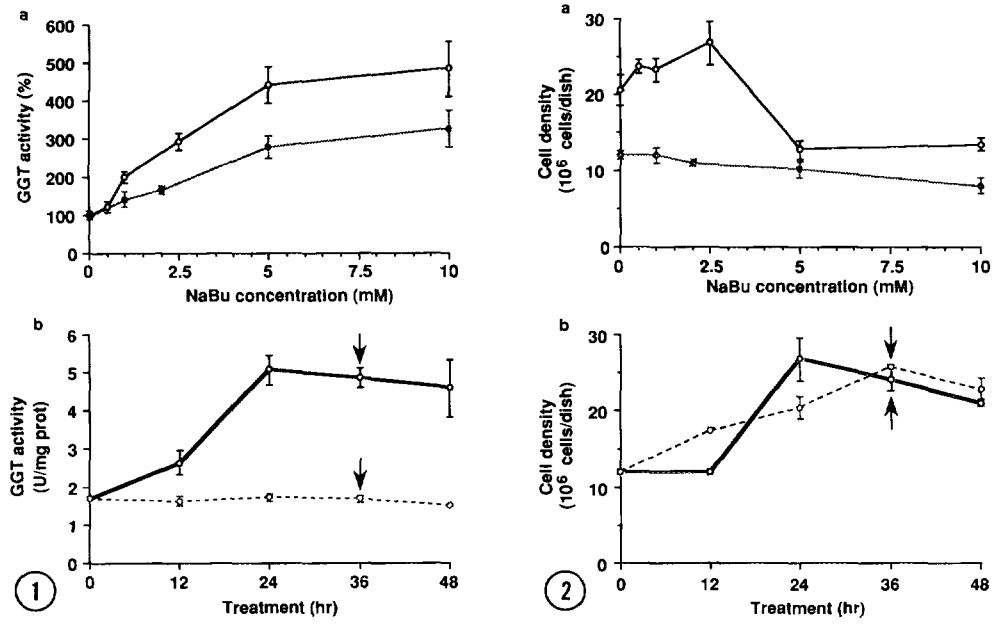


Fig. 1. Induction of recombinant GGT specific activity by NaBu
a. Influence of NaBu concentration on induction rates after 24-hr treatment in V79HGGT (open circles) and CHO-GGT (closed circles) cells. *GGT activity measured in the untreated cells was considered as 100%.*
b. Time course of GGT activity in V79HGGT cells cultured without (dashed line) and with (bold line) 2.5 mM NaBu. *The arrow indicates when the medium was renewed. Time 0 corresponded to the beginning of the treatment,* i.e., *48-hr growth without NaBu.*
Values reflect the mean and the standard deviation of three independent determinations.

Fig. 2. Effects of NaBu on the cell growth
a. Influence of the NaBu concentration on the density of V79HGGT (open circles) and CHO-GGT (closed circles) cells after 24 hr treatment.
b. Proliferation of V79HGGT cells throughout the culture in the absence (dashed line) or in the presence (bold line) of 2.5 mM NaBu. *The arrow indicates when the medium was renewed. Time 0 corresponded to the beginning of the treatment,* i.e., *48-hr growth without NaBu.*
Values reflect the mean and the standard deviation of three independent determinations.

by HPLC analysis. A more likely explanation could be a decline of the culture due to NaBu itself, as already reported for CHO cells (15). Thus, influence of NaBu was studied on the growth of our cell lines.

In the presence of high NaBu concentrations (5-10 mM), though no apparent toxic effect was generated by the inducer on the CHO-GGT cells, the viability of the V79HGGT cells was severely affected (Fig. 2a). So, each cell line clearly showed a different sensitivity to NaBu, which was also obvious when the cells were treated with lower NaBu concentrations ($\leq$ 2.5 mM): after 24 hr, whilst CHO-GGT cell growth was apparently not modified by the treatment, V79HGGT cell growth appeared to be induced. In the presence of 2.5 mM NaBu, cell density was observed to evolve in 3 successive steps throughout the culture (Fig. 2b): (1) a pause of cell growth at the beginning of the treatment; (2) a strong acceleration between 12 and 24 hr; (3) a final decrease of the cell density, probably describing the normal decline of a culture as observed even for the untreated cells. These data indicate that in the view of producing large quantities of recombinant human GGT, the conditions for induction must be determined by considering GGT biosynthesis and cell

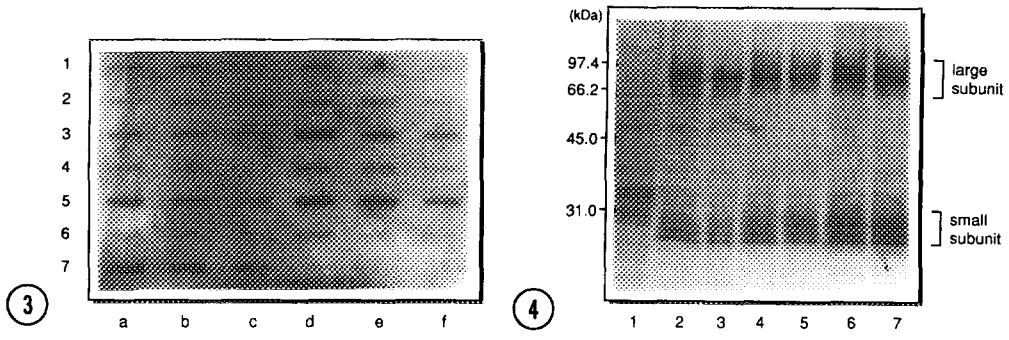


Fig. 3. Slot-blot analysis of total RNA fractions from V79HGGT cells
RNA samples from two separate preparations (A and B) were adsorbed on a nitrocellulose membrane and subjected to Northern blot using Hep G2 GGT cDNA as the probe. *Lanes a, b, c* and *d, e, f*: 10, 5, 2.5 µg of total RNA from preparations A and B, respectively. *Line 1*, 0 hr (beginning of the experiment); *2 and 3*, after 6 hr, without and with 2.5 mM NaBu; *4 and 5*, after 12 hr, without and with 2.5 mM NaBu; *6 and 7*, after 24 hr, without and with 2.5 mM NaBu.

Fig. 4. Immunoblot analysis of V79HGGT cell extracts
The cell extracts (30 µg of proteins) were subjected to SDS-PAGE and transferred to Immobilon-P membranes. GGT polypeptides were detected with an antiserum raised against human kidney GGT. *Lane 1*, parental V79 cells; *2-3*, untreated V79HGGT cells; *4-5*, V79HGGT cells treated with 2.5 mM NaBu for 24 and 36 hr; *6-7*, V79HGGT cells treated for 24 hr with 5 mM and 10 mM NaBu. Protein molecular masses were estimated in comparison with the following standards: rabbit muscle phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; hen egg white ovalbumin, 45.0 kDa; bovine carbonic anhydrase, 31.0 kDa.

density as a compromise. Therefore, we selected 2.5 mM NaBu for 48 hr and 5 mM NaBu for 24 hr as the optimal conditions for maximal GGT production in V79HGGT (13 U/dish) and CHO-GGT (8 U/dish) cells respectively, *i.e.* 0.5 U/$10^6$ cells in either line.

Molecular approach of the induction phenomenon. Since the inducibility of recombinant GGT activity was observed to be higher in V79HGGT cells, we investigated in greater detail the molecular mechanisms involved in this cell line. Total RNA samples were prepared from treated (with 2.5 mM NaBu) and untreated cells, and the relative amount of GGT specific mRNA in these samples was evaluated by slot blot (Fig. 3). GGT mRNA ratio was apparently not modified throughout the cell growth in the absence of NaBu (*lines 1, 2, 4 and 6*). On the other hand, treatment with NaBu increased this ratio as early as after 6 hr (*lane 3*), with a peak of induction estimated around 12 hr (*line 5*). These data clearly show that GGT mRNA induction came before that of GGT activity, suggesting that induction by NaBu is mediated through pretranslational mechanisms, most probably by enhancing transcription.

Characterization of the NaBu-induced enzyme. Since GGT is a membrane-bound heterodimeric enzyme, it was interesting to study in V79HGGT cells how the processing mechanisms would face such induction rates. Investigations were focused on the levels of induced proteins and some of their physico-chemical and catalytic properties. Immunoblot analysis of the cellular proteins showed that the induction of GGT activity resulted from an enhanced biosynthesis of the enzyme (Fig. 4), which can be correlated to the increased ratio of GGT mRNA observed earlier. Next, we observed that no GGT activity was measured in the culture medium, either with or without treatment with NaBu, demonstrating that all GGT polypeptides were anchored to membranes. Furthermore, NaBu did not apparently affect the heterodimeric structure of

Table 1. Comparison of apparent affinity constants of several human GGTs

| Source of GGT | $K_m$ GluCNA (mM) | $K_m$ Gly-Gly (mM) |
|---|---|---|
| V79HGGT (recombinant) | | |
| - uninduced | 0.85 ± 0.06 | 7.49 ± 0.07 |
| - NaBu-induced (1) | 0.87 ± 0.05 | 7.21 ± 0.12 |
| CHO-GGT (recombinant) | | |
| - uninduced | 0.70 ± 0.10 | 6.50 ± 0.50 |
| - NaBu-induced (2) | 0.71 ± 0.05 | 6.50 ± 0.80 |
| Hep G2 (endogenous) | 0.92 ± 0.01 | 10.79 ± 0.18 |
| Blood plasma | 1.01 ± 0.02 | 12.72 ± 0.10 |

(1) Induction with 2.5 mM NaBu for 48 hr.
(2) Induction with 5 mM NaBu for 24 hr.
Values represent the average of two or three independent determinations.

the induced GGT, since no signal corresponding to the uncleaved precursor was detected and the molecular masses of the two glycosylated subunits were similar to those previously estimated, 64-95 kDa and 27-31 kDa (5). Nonetheless, more glycoforms (which differ only in their sugar moiety), were visible in the samples after induction (Fig. 4, *lanes 4-7*), and could probably be visualized only because of the excessive amount of GGT loaded, as they were weakly or not detectable when GGT ratio in cellular proteins was lower. As a matter of fact, not only the profiles appeared identical when proteins equivalent to the same GGT activity were loaded rather than a same amount of proteins (data not shown), but both induced and uninduced pure GGTs also exhibited the same pattern of isoforms (Thioudellet *et al.*, manuscript in preparation).

The apparent affinity constants for GluCNA and Gly-Gly, the classical donor and acceptor substrates, were determined for human GGT in different cell extracts (Table 1). The induced recombinant GGTs from V79HGGT and CHO-GGT exhibited $K_m$ values which were very similar to those of the corresponding uninduced enzyme as well as those of the human hepatoma Hep G2 and human serum, suggesting that their catalytic properties were not significantly changed by NaBu.

DISCUSSION The present paper reports the induction by NaBu of a membrane-bound heterodimeric enzyme, namely GGT. The same cDNA, encoding the human hepatoma Hep G2 enzyme, was expressed in two separate transfected V79 and CHO Chinese hamster cell lines. When either cell line was cultured in the presence of millimolar concentrations of NaBu ($\leq$ 5 mM), recombinant GGT activity increased in a dose-dependent manner. We also showed that induction of GGT activity in V79HGGT cells by NaBu was mediated by an increase of GGT mRNAs and the subsequent elevation of the enzyme biosynthesis.

So, SV40 and cytomegalovirus immediate early promoters, which respectively control the expression of recombinant GGT in V79HGGT and CHO-GGT cells, should possess NaBu-responsive sequences located in their context and able to activate transcription. Such sequences apparently do not exist within the endogenous gene in Hep G2 cells as we failed to induce GGT activity under the same conditions in these cells, emphasizing the crucial role of the promoter/enhancer element in GGT expression induced by NaBu.

Characterization data on the NaBu-induced recombinant enzyme led us to conclude that increased GGT activity in the cells resulted from an enhanced biosynthesis of the enzyme, which exhibited catalytic and physico-chemical properties apparently close to those of its counterpart produced without NaBu. These results indicate that despite the important increase of GGT biosynthesis, maturation processes as well as targeting to the plasma membrane appeared to be complete. However, experiments are in progress in order to evaluate the effect of NaBu on the enzymes involved in the glycosylation steps and the possible modifications in the sugar chain moiety of the induced GGT.

These data are of obvious importance, providing us with very efficient expression systems and culture conditions allowing for high-scale production and easier purification of a recombinant enzyme that is currently used as a model for structure-function studies on human GGT.

Little is known about the molecular mechanism responsible for the effects of NaBu. This compound was demonstrated to cause histone hyperacetylation and, subsequently, gene activation (7). NaBu was also shown to reversibly arrest cell proliferation during the G1 phase (16-18). As already reported by Wright (15), NaBu-treatment increased the generation time of CHO-GGT cells. On the contrary, in V79HGGT cells, the cell growth was only transiently interrupted during the first 12 hr when treated with 2.5 mM NaBu, and it is interesting to note that the GGT mRNA ratio reached a maximum at this time (Fig. 2b & 3). It was more surprising to observe that these treated cells grew faster between 12 and 24 hr, whereas the GGT mRNA ratio had already decreased. These data suggest that recombinant GGT expression was maximal whilst NaBu exerted its antimitogenic action. Also, V79 and CHO cell growths seem to be differently sensitive to NaBu, possibly because of the characteristics of the V79 line which lacks a detectable G1 phase in its cell cycle (19). To our knowledge, this is the first report on such an effect of NaBu on cell growth. However, the transient arrest of cell growth observed during the first 12 hr of treatment could indicate that the proliferation inhibitory effect of NaBu could be mediated by additional minor targets different to those related to the G1 phase as proposed (18). Chalkley and Shires (20) reported that variant HTC cells exhibited an increased histone deacetylase activity, presumably allowing the cells to replicate in the presence of 6 mM NaBu. Since this paper is the first one stating treatment of V79 cells with NaBu, more experiments in these cells and others need to be carried out in order to know whether gene activation could be linked to a blocking of the cell cycle *via* the acetylation status of histones and other proteins.

ACKNOWLEDGMENTS

The authors are thankful to Dr. N. Strazielle for her help in performing RNA analysis. This work was supported by grants of the Community Bureau of Reference (Brussels, E.C.), the Institut de Biotechnologies de Nancy (funds from the District Urbain, Conseil Général and Conseil Régional de Lorraine, France), and the Fondation pour la Recherche Médicale (T.O.).

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