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Correlation Between Enhancing Effect of Sodium Butyrate on Specific Productivity and mRNA Transcription Level in Recombinant Chinese Hamster Ovary Cells Producing Antibody

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Abstract Sodium butyrate (NaBu) has been used to enhance protein expression levels in mammalian cell culture. To determine the clonal variability of recombinant Chinese hamster ovary (rCHO) cells in response to NaBu addition regarding specific antibody productivity (q_{Ab}), three rCHO clones were subjected to different concentrations of NaBu. For all three clones, NaBu addition inhibited cell growth and decreased cell viability in a dose-dependent manner. On the other hand, the enhancing effect of NaBu on q_{Ab} varied significantly among the clones. NaBu addition enhanced the antibody production of only one clone. RT-PCR analysis revealed that the changes in q_{Ab} correlated linearly with those of the mRNA transcription level. Thus, it was concluded that the different enhancing effects of NaBu on protein expression in rCHO cell clones resulted from their different mRNA transcription levels.

Keywords: Chinese hamster ovary cells, sodium butyrate, specific productivity, transcription level

Sodium butyrate (NaBu), a sodium salt of butyric acid, has been known to enhance specific genes controlled by some mammalian promoters including cytomegalovirus [3, 5, 14] and SV40 [7, 18, 19]. Thus, it has been extensively used in recombinant Chinese hamster ovary (rCHO) cells for the high-level expression of foreign proteins such as erythropoietin [4, 6, 13], β-interferon [17], antibody, factor VIII [6], interleukin-2 [9], nitric oxide synthase [14], and human follicle stimulating hormone [16].

Although the molecular mechanism responsible for the effects of NaBu has not yet been clearly elucidated, NaBu was demonstrated to loosen chromatin packing as a result of histone hyperacetylation, especially H3/H4, through inactivation of histone deacetylase [15]. The change in chromatin structure may facilitate access of DNA by transcription factors, and thereby increase transcription levels [22].

In CHO cells, the integration event of a transfected foreign gene in the host chromosomes occurs almost exclusively by nonhomologous recombination. Therefore, its integration positions are randomly distributed and can be further altered through methotrexate (MTX)-mediated gene amplification, increasing the clonal variability in a rCHO cell population [12]. Thus, as observed in a previous work based on the fluorescence of intracellular green fluorescence protein (GFP)-expressing cell lines [8], the response of rCHO cells to NaBu addition in regard to the specific productivity (q) of secreted foreign protein may vary among clones. However, this has not yet been clearly demonstrated.

In this study, we observed the clonal variation of rCHO cells in response to NaBu addition regarding q. Furthermore, in order to determine whether this clonal variation correlates with mRNA transcription level, RT-PCR analyses were performed with RNA isolated from three different rCHO cell clones subjected to different concentrations of NaBu.

rCHO cells producing a recombinant antibody were established, as described previously [21]. Briefly, parental CHO cells were made by transfection of a vector containing the dihydrofolate reductase (dhfr) gene and genes of heavy and light chains into dhfr-deficient CHO cells (DG44). Drug selection was carried out by seeding 10^3 cells per well in 96-well tissue culture plates (Nunc, Roskilde, Denmark) containing Iscove's modified Dulbecco's medium (IMDM; Invitrogen, Grand Island, NY, U.S.A.) supplemented with 10% (v/v) dialyzed fetal bovine serum (dFBS) and 500 μg/ml of G418 (all from Invitrogen). G418-resistant clones with high expression levels, which were determined by an enzyme immunosorbent assay (ELISA), were chosen for gene amplification and were subjected to successive rounds of selection in medium containing stepwise increments of MTX (0.005, 0.02, and 0.08 μM; Sigma, St. Louis, MO,

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U.S.A.). Three clones amplified at 0.08 μM MTX (C-08, C-11, and C-18) were chosen to examine the response to NaBu addition regarding q.

The medium for culture maintenance was IMDM supplemented with 5% (v/v) dFBS and 0.08 μM MTX. Cells were maintained as monolayer cultures in 25-cm^2 T-flasks (Nunc) in a humidified 5% CO_2 incubator at 37°C.

As demonstrated previously [11], immortalized mammalian cells are considered as heterogeneous even after very short periods of cultivation. Therefore, the term "clonally derived population" would be appropriate. However, throughout this article, we use the term "clone" for consistency.

Three clones (C-08, C-11, and C-18) were cultivated as monolayer cultures in 25-cm^2 T-flasks containing 5 ml of IMDM supplemented with 5% (v/v) dFBS and 0.08 μM MTX, in the presence of various concentrations (0–5 mM) of NaBu. Exponentially growing cells were seeded at a density of 1×10^5 cells/ml. After 3 days of cultivation, the spent medium was replaced with fresh medium containing the different concentrations of NaBu. Culture plates were sacrificed every day to determine cell concentration and viability. Culture supernatants were aliquoted and kept frozen at −70°C for the antibody assay.

Cell concentration was estimated using a hemacytometer (Sigma). Viable cells were distinguished from dead cells by the trypan blue dye exclusion method. The specific growth rate (μ) was based on data collected during the growth phase. Secreted antibody concentration was measured by sandwich ELISA, as described previously [10]. The specific antibody productivity (q_{Ab}) was based on the data collected during the growth phase and was evaluated from a plot of the antibody concentration against the time integral values of the growth curve [2, 20].

Fig. 1 shows cell growth, viability, and antibody production profiles. In the absence of NaBu, cell growth profiles of the three clones were similar. The μ of the three clones were 0.53–0.55/day in the absence of NaBu. For all three clones, NaBu addition inhibited cell growth and decreased cell viability in a dose-dependent manner, though the degree of growth inhibition and cell viability decrease differed slightly among the clones (Fig. 1A and 1B). C-08 was a little more resistant to NaBu addition than the other two clones.

On the other hand, antibody production profiles were quite different among the clones. When NaBu was added to the culture, only clone C-18 showed enhanced antibody

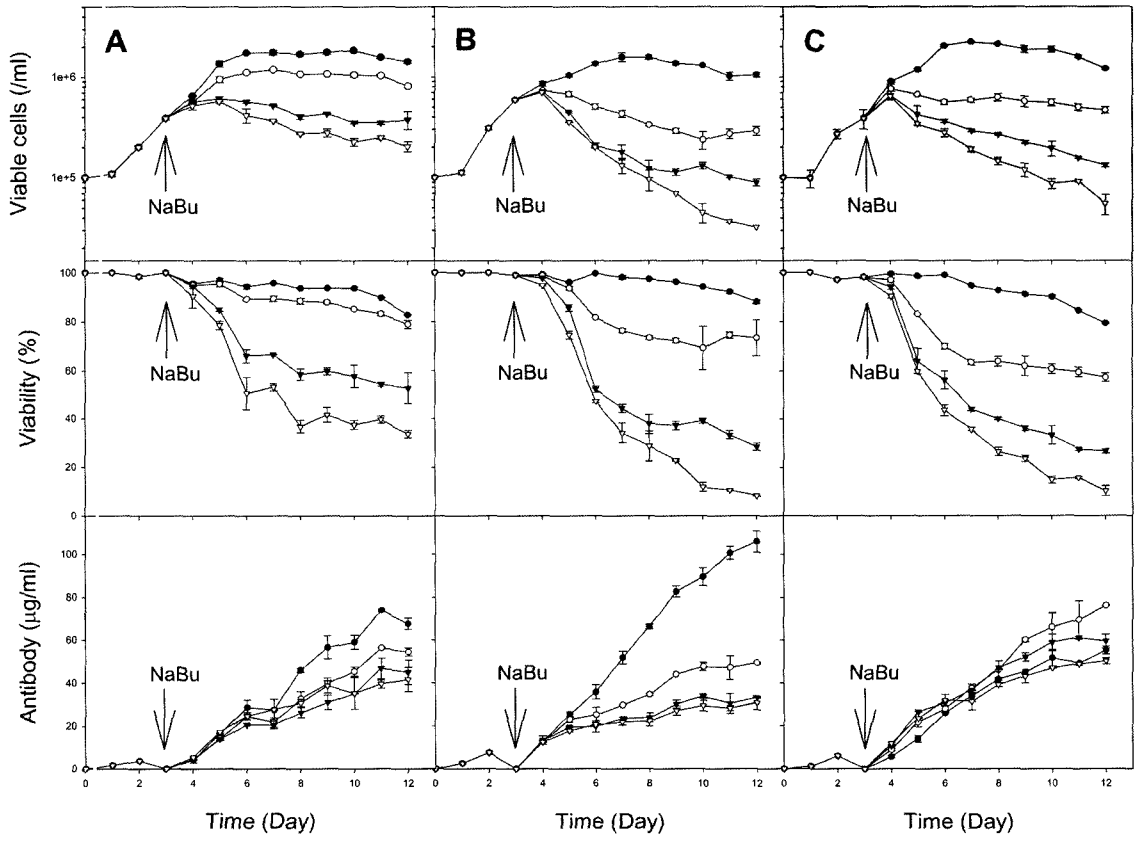

Fig. 1. Cell growth, viability, and antibody production in batch cultures with NaBu addition.
(**A**) C-08, (**B**) C-11, and (**C**) C-18. (●): 0 mM; (○): 1 mM; (▼) 3 mM; and (▽): 5 mM of NaBu addition. Arrows indicate the time of NaBu addition. The error bars represent the standard deviation calculated from the data obtained in duplicated experiments. The same experiment was repeated three times.

Table 1. Maximum antibody concentration and q_{Ab} at various culture conditions.[a]

NaBu (mM)	Maximum antibody concentration (μg/ml)			q_{Ab}[b] (μg/10^6 cells/day)		
	C-08	C-11	C-18	C-08	C-11	C-18
0	74.0±0.6	105.9±5.0	55.2±1.8	6.0±0.2	9.6±0.1	5.7±0.5
1	56.4±0.2	49.4±0.4	76.1±0.2	6.7±0.2	13.0±0.6	11.8±0.4
3	46.8±4.8	33.8±1.1	61.0±0.8	11.1±0.1	13.8±0.6	15.6±1.6
5	41.7±5.7	30.7±3.3	50.2±1.5	14.1±0.2	13.2±0.5	18.9±0.5

[a]Values are means±SD of duplicated experiments.
[b]The q_{Ab} was calculated from data in the range showing linear correlation between antibody concentration and time integral of viable cells after day 3.

production (Fig. 1C). By addition of 1 mM NaBu, the maximum antibody concentration was increased by 38%. Further increase in NaBu concentration did not result in increased antibody production because the cytotoxic effect of NaBu on cell growth outweighed its beneficial effect on q_{Ab}. In the C-08 and C-11 cultures, addition of even 1 mM NaBu significantly decreased antibody production. When considering tolerance against cytotoxicity from NaBu, clone C-18 was not any more tolerant than the other two clones. This result suggests that the q_{Ab} of clone C-18 was more significantly enhanced because of NaBu addition. In the C-18 culture, the use of higher concentrations of NaBu resulted in higher q_{Ab} values. By addition of 5 mM NaBu, the q_{Ab} was increased by 232%. In contrast, the q_{Ab} of clones C-08 and C-11 were increased by only 135% and 38%, respectively. Accordingly, the clonal variation of rCHO cells in response to NaBu addition regarding q_{Ab} was found to be significant.

The C-11 clone, which showed the highest q_{Ab} before NaBu addition, displayed the least enhancement in q_{Ab} by addition of NaBu. Previously, we had observed that the q of high-producing clones was not enhanced by lowering culture temperature as much as that of low-producing clones [21]. Thus, high-producing clones, which already have a relatively high transcription level, appear to have a limited capacity in q improvement by changing the culture environment, compared with low-producing clones.

The maximum antibody concentration and q_{Ab} of the three clones at various NaBu concentrations were summarized in Table 1.

In order to determine whether the clonal variation of rCHO cells in response to NaBu addition regarding q_{Ab} is related to the mRNA transcription level, the relative heavy and light chain mRNA contents were analyzed by RT-PCR analyses. RT-PCR analysis was performed three times with RNA samples prepared independently. Total RNA was isolated from cultured cells, which were harvested 2 days after NaBu treatment, using TRI reagent solution (Sigma) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μg of total RNA using the SuperScript synthesis system (Invitrogen). Heavy chain cDNA was amplified using the primer pair 5'-GAGAATTCACATTCACGATGTACTTG-3' and 3'-TGAAGGGGCTTGGCCAC-5' for 20 cycles (95°C for 50 s, 52°C for 50 s, and 72°C for 90 s). Light chain cDNA was amplified using the primer pair 5'-GCTGCAAAGCTTGGAAGCAAGATGGATTCA-3' and 3'-GACTTATTGAAGATAGGGTCTCTCCGG-5' for 17 cycles (95°C for 50 s, 52°C for 50 s, and 72°C for 90 s). Semiquantitative estimation of the RT-PCR product was accomplished by photodensitometric analysis of the bands in 1% agarose gel [1].

Fig. 2 shows the representative RT-PCR products in the three clones. The change in the band pattern was similar to that of q_{Ab}. In the case of clone C-18, the transcription level of both the heavy chain and light chain, like q_{Ab}, was increased with increasing NaBu concentration. The transcription level of clone C-08 treated with 1 mM NaBu was similar to that of the control. In addition, the transcription level of clone C-11 treated with different concentrations of NaBu was similar. This relationship between q_{Ab} and transcription level is clearly shown in Fig. 3. The q_{Ab} and transcription levels of the heavy and light chains of each clone treated with different concentrations of NaBu were normalized by those in the absence of NaBu. As shown in Fig. 3, the changes in the q_{Ab} of each clone induced by NaBu addition linearly correlated with those in the transcription levels, particularly of the heavy chain. Accordingly, the clonal variation of rCHO cells in response to NaBu addition regarding q appears to relate to the mRNA transcription level.

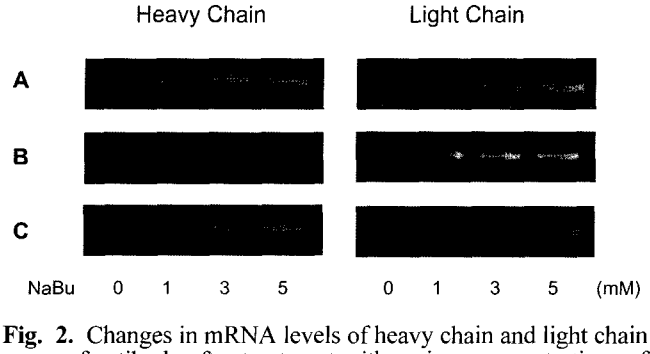

Fig. 2. Changes in mRNA levels of heavy chain and light chain genes of antibody after treatment with various concentrations of NaBu.
(**A**) C-08, (**B**) C-11, and (**C**) C-18. NaBu was added at day 3. Semiquantitative RT-PCR was performed to detect the mRNA of heavy chain and light chain genes. The experiments were repeated three times.

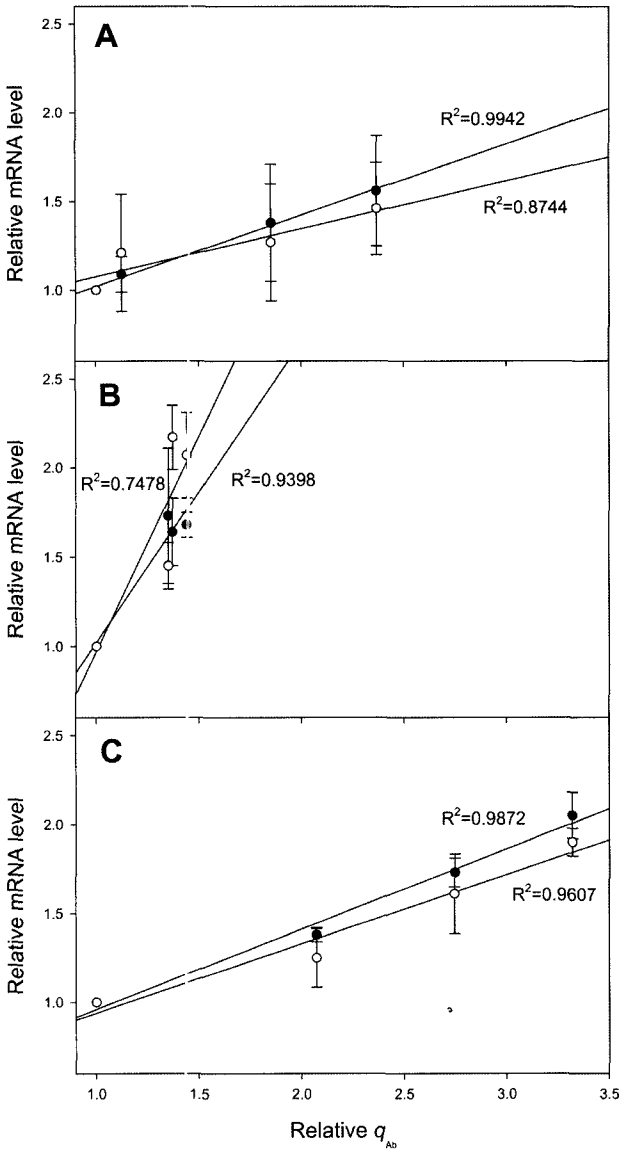

Fig. 3. Relationship between q_{Ab} and transcription level of the three clones.

(**A**) C-08, (**B**) C-11, and (**C**) C-18. The q_{Ab} and transcription levels of the heavy and light chains of each clone treated with different concentrations of NaBu were normalized by those in the absence of NaBu. (●): Heavy chain; (○): Light chain. Error bars represent the standard deviation (n=3).

The clonal variations in response to NaBu addition regarding q are indicative of the importance of clonal selection for manufacturing. If a culture process using NaBu addition is applied, the clone that shows enhanced q upon NaBu addition should be selected because of clonal variability.

In conclusion, it was found that the enhancing effect of NaBu on q of rCHO cells depends on the clone. It was also demonstrated that this clonal variation of rCHO cells in response to NaBu addition regarding q is related to the mRNA transcription level.

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