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n-Butyrate Enhances Induction of Thyroid Hormone-Responsive Nuclear Protein

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Abstract. Effects of n-butyrate on nuclear thyroid hormone receptors and on thyroid hormone-responsive nuclear protein were investigated by means of a perfusion system in rat liver. Treatment with 5 mM n-butyrate resulted in an increase (150%) in the maximal binding capacity of 3,5,3'-L-triiodothyronine (T_3) nuclear receptors without altering the affinity of receptor for T_3. However, further perfusion for 4 h decreased the number of the receptors to the control level. n-Butyrate increased the amount of acetylated histone H4. The ability of nuclear T_3 receptors to bind to core histones was diminished by acetylation of the core histones. Thyroid hormone-responsive nuclear protein (n protein) was increased by T_3. The induction of the n protein by T_3 was augmented by n-butyrate. These results suggested that n-butyrate modulates thyroid hormone-responsive gene expression in rat liver via the increased number of nuclear receptors or changes in the chromatin constitution.

Key words: n-Butyrate, Thyroid hormone action, Histone acetylation.

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POST-SYNTHETIC modifications of histones may play an important role in transcriptional regulation [1–5]. All reversible histone acetylations are located within the N terminal basic domains of the core histones H2A, H2B, H3, and H4 [6]. Acetylation of the lysine residues of H3 and H4 may induce supercoil of the gene and modulate transcription [7]. Samuels *et al.* reported that n-butyrate reduces the levels of thyroid hormone nuclear receptors in GH1 cells possibly by inhibiting the deacetylation of histones [8]. They found that n-butyrate increased the levels of prolactin and growth hormone mRNA [9]. On the other hand, n-butyrate increases the levels of thyroid hormone nuclear receptors in non-pituitary cells, such as rat hepatoma cells (H35) [10] and rat glioma cells (CL6) [11]. It therefore seems that n-butyrate induces cell specific modulation of nuclear thyroid hormone receptors.

In this study we examined the effect of n-butyrate on the levels of nuclear thyroid hormone receptors in rat liver. We also measured the levels of thyroid hormone responsive rat hepatic protein [12, 13] in order to investigate whether the modification of the receptors by n-butyrate alters thyroid hormone action.

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Materials and Methods

Materials

[^{125}I]triiodo-L-thyronine ([^{125}I]T_3) (3400 μCi/μg) was obtained from New England Nuclear (Boston, MA). Sodium n-butyrate and unlabeled T_3 were obtained from Sigma Chemical Co. (St. Louis, MO). Histones were purchased from Boehringer Mannheim (Mannheim, Germany). William's medium E was obtained from Flow

Laboratories (McLean VA). Dithiothreitol (DTT) and silver nitrate were obtained from Nakarai Chemical Co. (Kyoto, Japan). Peroxidase-conjugated antirabbit immunogloblin G (heavy chain and light chain) was obtained from Cappel Laboratory (Cochroranville, PA). CNBr-activated Sepharose 4B was obtained from Pharmacia Fine Chemical Co. (Rockland, IL).

Perfusion of rat liver

Male Wister rats (200g) were rendered hypothyroid by throidectomy as described before [12]. The serum levels of T_3 and T_4 (0.4 nmol/*l* and 6.4 nmol/*l*, respectively) were markedly lower in the thyroidectomized animals than those (1.5 nmol/*l* and 70.8 nmol/*l*, respectively) in control (sham-operated) rats. Perfusion of the liver was performed according to the method of Sugano *et al.* [14]. The livers were perfused for 1-h with William's E medium which did not contain n-butyrate. The right lobes of the perfused livers were then excised. Perfusion of the left and caudal lobes was then done with William's E medium in the absence (control) or presence of 5 mM n-butyrate. Two and 6-h after the beginning of the perfusion, the left and caudal lobes were removed. The excised tissues were stored at −70°C until used.

Preparation of nuclear extract

Rat liver nuclei were prepared as described [15]. The nuclei which had been perfused with n-butyrate were prepared in the presence of 5 mM n-butyrate. The protein/DNA ratio of the nuclei was 2.7. Nuclear extracts which contained nuclear T_3 receptors were prepared as described previously [15].

Estimation of n-protein

The amount of T_3 responsive nuclear protein (n protein) was quantitated by Western blotting with anti n protein antibody as described before [12].

Preparation of histones

Rat liver core histones were purified according to the method described previously [17]. Nuclei were extracted with 5% trichloroacetic acid (TCA) to obtain H1 histone. After extraction with TCA, the nuclei were further extracted with 0.25 M HCl to obtain core histones. When histones were prepared from n-butyrate-treated liver, 5 mM n-butyrate was included in the extraction buffer. After extensive dialysis against distilled water, the histones were usually concentrated by lyophirization because the recovery of histones by lyophirization was higher than that by ethanol precipitation. Electrophoresis of histones with discontinuous acetic acid-urea-Triton X-100 gel was performed as described by Bonner *et al.* [18]. The gels were stained with Coomassie Brilliant Blue-R250 and scanned at 550 nm in Shimadzu spectrophotometer.

T_3 binding to nuclear receptors

Assay of nuclear T_3 binding was performed as described previously [19]. The nuclei were suspended in 0.25 M sucrose, 1 mM $MgCl_2$, 20 mM Tris-HCl pH 7.85, 1 mM dithiothreitol (DTT) (SMTD buffer). The nuclear suspension containing 10–25 μg DNA was incubated for 2 h at 22°C with various concentrations of T_3 (0–0.3 nM) and 30 pM of [^{125}I]T_3 in a total volume of 0.5 m*l* of SMTD buffer. After the incubation, the nuclei were immediately cooled in an ice bath and subsequent procedures were performed at 2°C. The nuclei were collected by centrifugation, and the supernatant (0.4 m*l*) was kept in an ice bath to determine the number of the receptors released during incubation [20]. The nuclei were, thereafter, washed twice with SMTD buffer containing 0.25% Triton X-100, and radioactivity was counted. For determination of the number in released receptors, 0.4 m*l* of Dowex 1 × 8, Cl, 200 to 400 mesh anion exchange resin in SMTD containing 0.8 M KCl was added to the 0.4 m*l* supernatant to separate bound and free hormones. After the centrifugation, an aliquot of supernatant was removed and radioactivity was counted for bound hormone determination.

Binding assay of [^{125}I]T_3-receptor complex to core histone-Sepharose

Core histone-Sepharose was prepared by coupling purified core histone to CNBr-activated Sepharose 4B [21]. For the binding assay, [^{125}I]T_3-labeled receptor and histone-Sepharose were incu-

bated at 2°C with 0–200 μg of histones prepared from n-butyrate or vehicle perfused liver in 90 mM KCl, 1 mM EDTA, 0.3 mM $MgCl_2$, 10 mM Tris HCl, pH 8.0 and 1 mM DTT in glass tubes (0.2 m*l* total volume) [21]. During the incubation, the assay tubes were shaken every 5 min. After the incubation, 1 m*l* of ice cold 50 mM NaCl, 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA were added. The tubes were vortexed and centrifuged at 1,000 × g for 10 min. The pellet was washed with 1 m*l* of the same buffer, and the radioactivity was determined. Parallel tubes containing plain ethanolamine-blocked Sepharose were always included to determine non-specific binding. Binding of [^{125}I]T_3-receptor to histones was calculated by subtracting non-specific binding from total binding.

Miscellaneous

The protein concentration was measured by the method of Lowry *et al.* with bovine serum albumin as the standard [22]. The DNA concentration was measured by the method of Burton with calf thymus DNA as the standard [23]. Statistical significance of the difference between two groups was assessed by Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

Results

Effect on n-butyrate on thyroid hormone-responsive rat hepatic nuclear protein

In order to examine the effect of n-butyrate on the induction of n-protein by T_3, hypothyroid rat livers were perfused with or without n-butyrate and/or T_3 for 6 h. The amount of n protein was estimated by Western blotting. When thyroidectomized rat liver was perfused, 100 nM of T_3 markedly increased the amount of rat hepatic n-protein. Induction of the n protein was enhanced when 5 mM n-butyrate was present in the perfusate (Fig. 1).

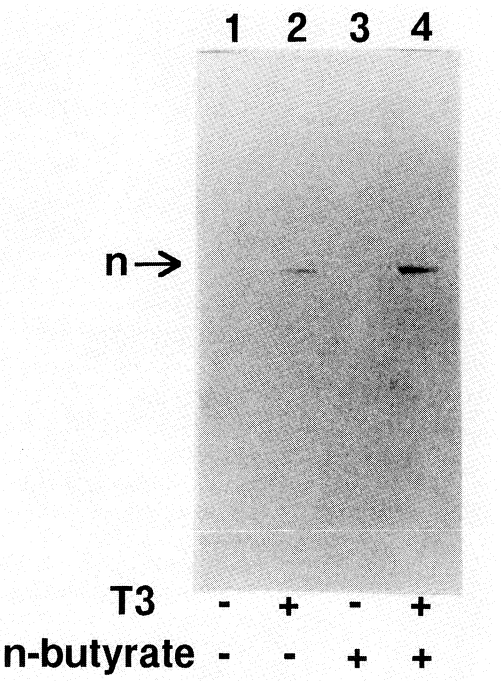

Fig. 1. Effect of n-butyrate on the induction of the n protein by T_3 in rat liver. After perfusion with control medium for 1-h, perfusion was further continued for 6-h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 nM T_3 with (lanes 3 and 4) or without (lanes 1 and 2) 5 mM n-butyrate. After perfusion, nuclear extracts were prepared. Nuclear extracts (50 μg protein) were applied to SDS-PAGE and the n protein was detected by Western blotting with the anti n protein antibody (IgG). Two other experiments gave similar results.

Effect of n-butyrate on the levels of rat hepatic nuclear T_3 receptors

We next evaluated the effect of n-butyrate on rat hepatic nuclear T_3 receptors by T_3 binding assay. Because nuclear receptor was released into the incubation medium during the assay, the released receptor as well as the chromatin-associated receptor was measured by Scatchard analysis (Fig. 2). Bernal *et al.* reported that as the receptor release is higher in the presence of EDTA which would inhibit Ca^{2+} and Mg^{2+}-dependent endonucleases, the receptor release seems to be dependent upon the integrity of the nuclear membrane [20]. Affinity constants (ka) treated without n-butyrate (open circles) were 0.8×10^9 liter/M (Panel A) and 1.0×10^9 liter/M (panel B) for chromatin associated receptors and released receptors, respectively, whereas those treated with n-butyrate (closed circles) were 0.8×10^9 liter/M (Panel A) and 1.1×10^9 liter/M (Panel B), respectively. n-Butyrate did not significantly affect the affinity for T_3 or maximal T_3 binding capacity

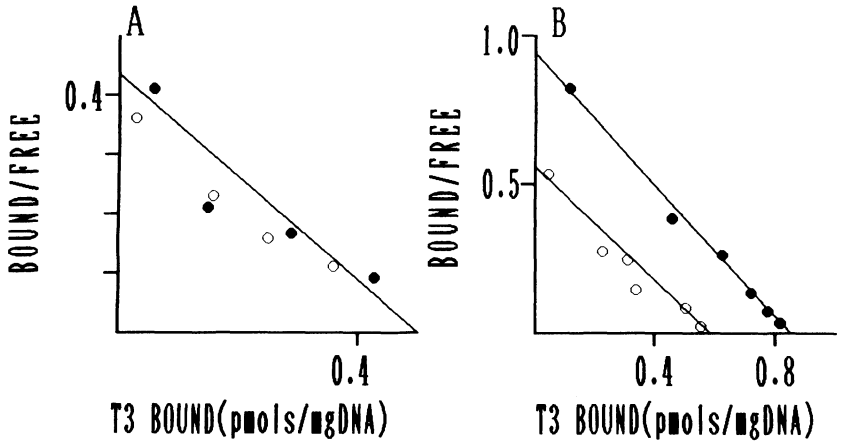

Fig. 2. Scatchard analysis of T_3 binding to nuclear receptors. Nuclear receptors prepared from rat livers which had been perfused with control medium (open circle) or with medium containing 5 mM n-butyrate (closed circle) for 2-h were incubated with [^{125}I]T_3 (30 pM added) in the presence of various concentrations of unlabeled T_3 for 2-h at 18°C. After incubation the mixture was centrifuged at 1,000 × g at 2°C, and the [^{125}I]T_3 bound to the receptors was measured in the nuclear pellet (Panel A) and in the supernatant (panel B). Each point indicates the mean of triplicate assays. In two additional experiments, similar results were obtained.

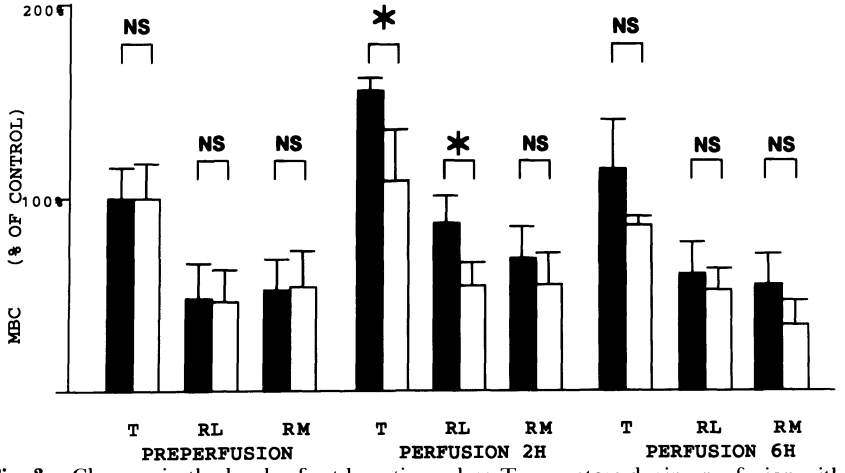

Fig. 3. Changes in the levels of rat hepatic nuclear T_3 receptors during perfusion with n-butyrate. After preperfusion with control medium for 1-h, livers were further perfused for 2 or 6 h in the absence (open bar) or presence (closed bar) of 5 mM n-butyrate. After the perfusion, nuclear T_3 bindings in the supernatant (RL) and pellet (RM) were assessed by Scatchard analysis. Total MBC (T) indicates the sum of the receptors in the nuclear pellet and in the supernatant. Each value is the mean ± SD of four determinations. One hundred percent indicates the MBC value in the right lobes which were preperfused with William's E alone for 1-h. In two additional experiments, similar results were obtained. *$P<0.05$ compared with MBC of the liver perfused with control medium for 2-h after 1 h preperfusion.

(MBC) of the receptors associated with the nuclear pellet. However, the MBC of the released receptors was higher in the n-butyrate-treated rat liver than in the control, whereas the affinity for T_3 was not affected. As shown in Fig. 3, the total MBC (MBC in the nuclear pellet + MBC recovered in the incubation medium) was significantly increased to 150% after perfusion for 2 h with n-butyrate. The levels of nuclear receptors, however, were decreased to the control level after

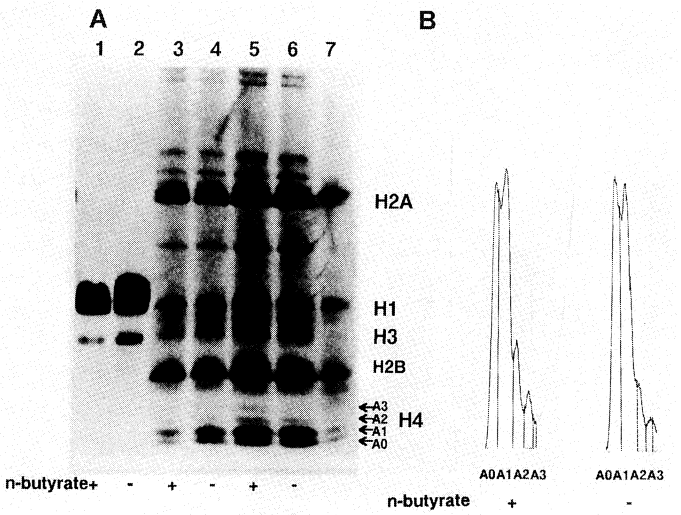

Fig. 4. Analysis of histones by polyacrylamide gel electrophoresis. Histones obtained from liver which was perfused in the absence (lanes 2, 4, 6) or presence (1, 3, 5) of 5 mM n-butyrate for 6 h were analyzed electrophoretically on acid urea Triton polyacrylamide gels (Panel A). H1 histone was extracted by 5% TCA (lanes 1 and 2) and core histones by 0.25% HCl (lanes 3–6). They were then concentrated with ethanol (lanes 3 and 4) or by lyophyrization (lanes 5 and 6). Standard histones which were purchased were applied to lane 7. The gels were stained with Coomassie Brilliant Blue R-250 and lanes 5 and 6 were scanned at 550 nm in a Shimadzu spectrophotometer (Panel B). A0, A1, A2, A3 indicates non-, mono-, di-, tri-acetylated H4 histone, respectively.

perfusion of the liver for 6 h.

Effect of n-butyrate on the acetylation of histones

Acetylated histones were analyzed by acid-urea-Triton × 100 polyacrylamide gel electrophoresis (Fig. 4A). H4 Histone (lane 5 and 6) from perfused liver was scanned in a spectrophotometer in order to estimate the histone acetylation. Relative amounts of tri-acetylated, bi-acetylated, mono acetylated and non-acetylated H4 histones were 6.4%, 13.0%, 52.8%, 27.8% of the total H4 histone, respectively, in n-butyrate treated liver, whereas those of control liver were 3.3%, 7.2%, 49.7%, 39.8%, respectively. Tri, bi and mono-acetylated histones were increased in the presence of n-butyrate (Fig. 4B).

Nuclear receptor-T_3 complex binding to histones

When nuclear T_3 receptor [^{125}I]T_3 complex was incubated with the histone-Sepharose in the presence of core histones prepared from liver which had been perfused with or without n-butyrate, core histone prepared from n-butyrate-treated rat liver displaced the receptor-[^{125}I]T_3 complex binding to histone-Sepharose less potently than those prepared from control liver (Fig. 5).

Discussion

Previous studies showed that rat hepatic n-protein was increased after the administration of T_3 to thyroidectomized rats [12, 13]. In this study, the effect of T_3 on n protein was evaluated by perfusion of rat liver followed by Western blotting. The amount of n protein was increased after the addition of 100 nM of T_3 in the perfusate. The induction of n protein was enhanced when 100 M T_3 and 5 mM n-butyrate was simultaneously added to perfusate. There are several possible mechanisms by which n-butyrate enhanced the effect of T_3 on the n protein. n-Butyrate is known to induce acetylation of core histones, activate some gene expression, inhibit cell growth and alter

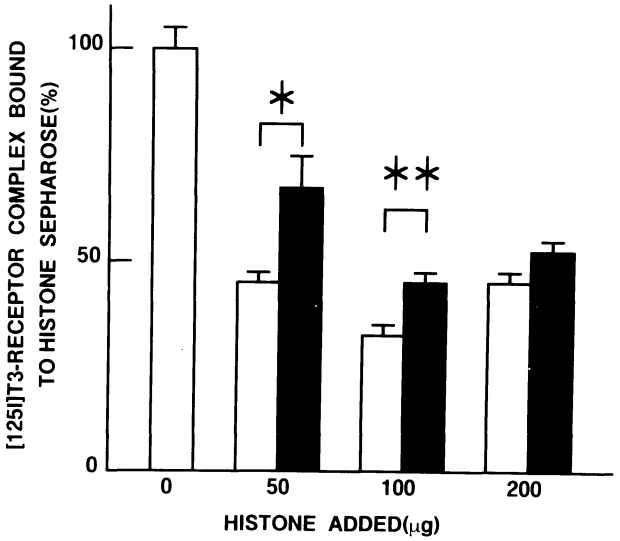

Fig. 5. Effect of treatment with n-butyrate on core histone-binding to [^{125}I]T_3-nuclear receptor complex. Core histones were prepared from rat liver which had been perfused without (open bar) or with 5 mM n-butyrate (closed bar) for 6-h. Fifty fmol [^{125}I]T_3-receptor complex was incubated for 30 min at 2°C with core histone-Sepharose (0.1 mg protein) in the presence of various concentrations of core histone prepared from perfused liver. After incubation the core histone-Sepharose was recovered by centrifugation and was washed with the buffer twice. The radioactivity bound to the core histone-Sepharose was measured. Specific [^{125}I]T_3 receptor binding to core histone was calculated by subtracting non-specific binding and was expressed as % of binding in the absence of free histones. Each value indicates the mean ± SD of three determinations. In two additional experiments, similar results were obtained. *$P<0.05$, **$P<0.005$ compared with histones prepared from rat liver which had been perfused with William's E alone (open bar).

the structure of chromatin [24]. In fact acetylation of histones by n-butyrate induces unfolding of DNA [7] and changes the chromatin structure, which consequently increases the accessibility of transcription factors to their responsive elements [25]. It is therefore possible that n-butyrate enhances T_3 action at the nuclear level via changes in chromatin structure, although further studies are required to confirm this.

It was shown previously that core histones bind nuclear receptors [21]. The present study showed that core histones prepared from n-butyrate-treated liver bound less tightly to nuclear T_3 receptor complex. We previously demonstrated that H4 histone bound to the nuclear receptor most strongly among subfractions of core histones [26]. The present results agreed with previous reports by others in that treatment with n-butyrate promoted the acetylation of H4 histone [8, 10]. It is therefore possible that acetylated H4 histone binds less strongly to the receptors than unacetylated H4 histone and that receptors dissociate from core histone after treatment with n-butyrate. This could also be the reason for the higher rate of release of the receptors from nuclei during the binding assay. Thyroid hormone action could possibly be modified by such an alteration in the localization of the receptors in nuclei as well as changes in chromatin structure elicited by treatment with n-butyrate.

Another possibility was that the transient increase in the level of nuclear receptors enhanced the thyroid hormone action. However, n-butyrate-induced increase in the level of nuclear receptors was not so great as enhancement by n-butyrate on thyroid hormone dependent n protein induction, suggesting that the other factors may be also involved. The increase in the number of nuclear receptors by n-butyrate was also reported by Mitsuhashi *et al.* and they found that the increase in the receptor was due to the prolongation of its half life [10]. The increase in the receptor caused by n-butyrate was also observed in rat glioma cells [11], rat hepatoma cells and human skin fibroblasts [10]. Samuels *et al.* reported that n-butyrate reduced the number of nuclear thyroid hormone receptors in GH1 cells which were derived from rat pituitary tumor [8]. Further Lazar found that n-butyrate abolished the induction of growth hormone gene expression by T_3, which was associated with the reduction in T_3 receptor (beta) mRNA in GH3 cells [27]. So the effect of n-butyrate on the nuclear T_3 receptors and on the T_3 action seems to differ according to the type of tissue.

References

1. Allfrey VG (1980) Molecular aspects of the regulation of eukaryotic transcription-nucleosomal proteins and their postsynthetic modifications in the control of DNA comformation and template function. In: Goldstein L, Presscott DM (eds) Cell Biology: A Comprehensive Treatise. Academic Press, New York, vol 3: 347–437.
2. Chahal S, Matthews HR, Bradbury EM (1980) Acetylation of histone H4 and its role in chromatin structure and function. *Nature* 287: 76–79.
3. Allegra P, Sterner R, Clayton DF, Allfrey VG (1987) Affinity chromatographic purification of nucleosomes containing transcriptionally active DNA sequences. *J Mol Biol* 196: 379–388.
4. Waterborg JH, Matthews, HR (1984) Pattern of histone acetylation in Physarum polycephalum. *Euro J Biochem* 142: 329–335.
5. Zahng DE, Nelson DA (1988) Histone acetylation in chicken erthrocytes. Rates of acetylation and evidence that histones in both active and potentially active chromatin are rapidly modified. *Bisochem J* 250: 233–240.
6. Doenecke D, Gallwitz D (1982) Acetylation of histones in nucleosomes. *Mol Cell Biochem* 44: 113–128.
7. Norton VG, Imai BS, Yau P, Bradbury EM (1989) Histone acetylation reduces nucleosome core particle linking number change. *Cell* 57: 449–457.
8. Samuels HH, Stanley F, Casanova J, Shao TC (1980) Thyroid hormone nuclear receptor levels are influenced by the acetylation of chromatin-associated proteins. *J Biol Chem* 255: 2499–2508.
9. Stanley F, Samuels HH (1984) n-Butyrate effects thyroid hormone stimulation of prolactin production and mRNA levels in GH1 cells. *J Biol Chem* 259: 9768–9775.
10. Mitsuhashi T, Uchimura H, Takaku F (1987) n-Butyrate increases the level of thyroid hormone nuclear receptor in non-pituitary cultured cells. *J Biol Chem* 262: 3993–3999.
11. Oritiz-Caro J, Montiel F, Pascaul A, Aranda A (1986) Modulation of thyroid hormone nuclear receptors by short-chain fatty acids in Glial C6 cells. *J Biol Chem* 261: 13997–14004.
12. Bernal J, Cleoni AH, DeGroot LJ (1978) Triiodothyronine stimulation of nuclear protein synthesis. *Endocrinology* 102: 452–459.
13. Miyamoto T, Ichikawa K, Hashizume K, Nishii Y, Takeda T, Kobayashi M, Suzuki S, Yamada T (1991) Purification and characterization of thyroid hormone-responsive rat hepatic protein. *Endocrinology* 129: 907–914.
14. Sugano T, Suda K, Shimada M, Oshino N (1978) Biochemical and ultrastructural evaluation of isolated rat liver systems perfused with a hemoglobin-free medium. *J Biochem* 83: 995–1007.
15. Ichikawa K, DeGroot LJ (1986) Separation of DNA-binding domain from hormone- and core histone-binding domains by trypsin digestion of rat liver nuclear thyroid hormone receptor. *J Biol Chem* 261: 16540–16546.
16. Bernal J, DeGroot LJ (1977) Thyroid hormone receptors: Release of receptor to the medium during in vitro incubation of isolated rat liver nuclei. *Endocrinology* 100: 648–658.
17. Eberhardt NL, Ring JC, Johnson LK, Lathan KR, Apriletti JW, Kitsis RN, Baxter JD (1979) Regulation of activity of chromatin receptors for thyroid hormone: possible involvement of histone like protiens. *Proc Natl Acad Sci USA* 76: 5005–5009.
18. Bonner WM, West MHP, Stedman JD (1980) Two-dimensional gel analysis of histones in acid extracts of nuclei, cells, and tissues. *Euro J Biochem* 109: 17–23.
19. Ichikawa K, Hughes IA, Horwitz AL, DeGroot LJ (1987) Characterization of nuclear thyroid hormone receptors of cultured skin fibroblasts from patients with resistance to thyroid hormone. *Metabolism* 36: 392–399.
20. Bernal J, DeGroot LJ (1977) Thyroid hormone receptors: Release of receptor to the medium during in vitro incubation of isolated rat liver nuclei. *Endocrinology* 100: 648–655.
21. Ichikawa K, Bentley S, Fee M, Degroot LJ (1987) Modification of deoxyribonucleic acid-thyroid hormone receptor interaction by histones. *Endocrinology* 121: 893–899.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 266–275.
23. Burton K (1956) Study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 62: 315–319.
24. Kruh J (1982) Effects of sodium butyrate, a new pharmacological agent, on cells in culture. *Mol Cell Biochem* 42: 65–82.
25. Lee DY, Hayes JJ, Pruss D, Wolffe AP (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. *Cell* 72: 73–84.
26. Sakurai A, Ichikawa K, Hashizume K, Miyamoto T, Yamauchi K, Ohtsuka H, Nishii Y, Yamada T (1989) Possible role of histones in the organization of rat liver thyroid hormone receptors in chromatin. *J Endocrinol* 121: 337–341.
27. Lazar MA (1990) Sodium butyrate selectively alters thyroid hormone receptor gene expression in GH3 cells. *J Biol Chem* 265: 17474–17477.