Using cultured GH₁ cells, a growth hormone and prolactin-producing rat pituitary cell line, we have shown that n-butyrate and other short chain carboxylic acids stimulate histone acetylation and elicit a reduction of thyroid hormone nuclear receptor which is inversely related to the extent of acetylation (Samuels, H. H., Stanley, F., Casanova, J., and Shao, T. C. (1980) J. Biol. Chem. 255, 2499-2505). In this study, we compared the n-butyrate and propionate modulation of receptor levels to regulation of the growth hormone and prolactin response by 3,3',5'-triiodo-L-thyronine (L-T₃). n-Butyrate (0.1-10 mM) did not stimulate growth hormone production. L-T₃ stimulated the growth hormone response 4- to 5-fold and n-butyrate (0.5–1 mM) increased L-T₃ stimulation of growth hormone production 1.5- to 2-fold compared to L-T₃ alone. L-T₃ stimulation of growth hormone production at higher n-butyrate concentrations decreased in parallel with the n-butyrate-mediated reduction of receptor levels. In contrast with the growth hormone response, n-butyrate (0.5 mM) increased basal prolactin production about 5-fold. Prolactin production, which is inhibited 25 to 50% by L-T₃, was stimulated between 20- and 70-fold by L-T₃ + n-butyrate (0.5–1 mM) and this decreased at higher n-butyrate levels. Prolactin mRNA and growth hormone mRNA levels paralleled the changes in prolactin and growth hormone production rates. These effects of L-T₃, n-butyrate, or L-T₃ + n-butyrate appeared unrelated to changes in cAMP levels or global changes in DNA methylation of the growth hormone or prolactin genes. Propionate elicited the same effects as n-butyrate but at a 5- to 10-fold higher concentration consistent with their relative effect on stimulating acetylation of chromatin proteins. These results suggest that prolactin gene expression is under partial regulatory repression which is reversed by a carboxylic acid-mediated postsynthetic modification event which allows for stimulation of the prolactin gene by thyroid hormone.

GH₁ cells are a useful cell model to study the mechanism of thyroid hormone action in cultured cells (1-6). In GH₁ cells, L-T₃ inhibits prolactin production by 25 to 50% (1) and stimulates a 3- to 10-fold increase in growth hormone synthesis and mRNA accumulation (2-4). Stimulation of growth hormone mRNA levels by L-T₃ appears to solely reflect increased growth hormone gene transcription rates (7-9). Abundant evidence indicates that these responses are mediated by the binding of hormone to a nuclear associated receptor (4, 5). The receptor is an intrinsic chromatin-associated protein (8,000 receptors/cell nucleus) with a half-life of about 5 h and a synthetic rate of approximately 1,800 molecules/cell/h (6). DNase II digestion studies suggest that a subset of the receptor is concentrated in transcriptionally active chromatin in GH₁ cells (5).

Studies in intact cells and isolated nuclei have shown that n-butyrate increases the level of histone and nonhistone protein acetylation by inhibiting the rate of deacetylation rather than altering the rate of acetylation (10, 11). This results from the n-butyrate inhibition of a ubiquitous chromatin-associated deacetylase(s) (12). This effect is not unique to n-butyrate but also occurs with other short chain aliphatic carboxylic acids with the potency of n-butyrate > propionate = valerate > acetate (13). Since a variety of studies in other systems suggested a relationship between histone acetylation and "gene" activation, we previously examined the effect of n-butyrate on thyroid hormone nuclear receptor levels and excision from chromatin by nuclease digestion (14). We found that n-butyrate elicited a decrease of thyroid hormone receptor abundance which was inversely related to the extent of histone acetylation. The reduction of receptor levels occurred over the same concentration range at which n-butyrate inhibited a chromatin-associated deacetylase in GH₁ cells. Other carboxylic acids also elicited a reduction of receptor which was directly related to the extent of inhibition of deacetylase activity as reflected in the extent of histone acetylation (14).

Yen and Tashjian (15) have recently shown that n-valerate and n-butyrate can increase growth hormone and prolactin production in GH₁ cells. In this paper, using GH₁ cells, we have examined the relationship between the carboxylic acid alteration of thyroid hormone receptor levels and the L-T₃ regulation of growth hormone and prolactin production and mRNA abundance. With low concentrations of n-butyrate (0.5-1.0 mM), L-T₃ stimulation of growth hormone production was 1.5- to 2-fold greater than with L-T₃ alone. At higher n-butyrate concentrations, L-T₃ stimulation of the growth hormone response decreased in parallel with the carboxylic acid-mediated reduction of receptor. With 0.5-1.0 mM n-butyrate, L-T₃ markedly stimulated rather than inhibited prolactin production and mRNA levels. This response also decreased in parallel with the carboxylic acid-mediated decrease of thyroid hormone receptor levels. These studies suggest that partial repression of prolactin gene expression is reversed by a carboxylic acid-mediated postsynthetic modification event. Low concentrations of carboxylic acid, which do not decrease
thyroid hormone receptor levels, appear to allow for positive control of the prolactin gene by thyroid hormone.

**EXPERIMENTAL PROCEDURES**

**Materials**—L-[3,4-14]T3 (1200 μCi/μg), immunoreagents for cAMP determination, and DNA polymerase I were from New England Nuclear. [α-32P]dCTP and [3H]iodide (carrier-free) were from Amer sham. Triton X-100 and propionate were from Eastman. n-butyrate was from Sigma, and all cell culture media and sera were from Gibco. Anaerobic culture (AG-1-XS) was from Bio-Rad. oligo(dT)-cellulose (Type 3) was from Collaborative Research. Proteinase K was from Beckman, SeaKem LE-agarose was from FMC, and forskolin was from Calbiochem-Behring. DNase I was from Worthington and all restriction endonucleases were from New England Biolabs. Rat growth hormone and prolactin for iodination, reference standards, and antisera for radioimmunoassay determination were obtained through the generosity of the Rat Pituitary Hormone Program of the National Institutes of Health. pRGH-1, a rat growth hormone cDNA-bearing plasmid, was generously provided by John D. Baxter (University of California, San Francisco). pPRL-2, a rat prolactin cDNA-bearing plasmid, was generously provided by Richard A. Maurer (University of Iowa, Iowa City). Both plasmids were propagated and amplified in bacteria according to standard procedures (18). All other reagents were of the highest purity available and were obtained from Sigma, British Drug House, Pharmacia, or Fisher Scientific.

**Cell Culture Conditions and Estimation of Growth Hormone and Prolactin Production Rates**—GH1 cells were grown in monolayer culture in 1-cm² multiwell dishes, 25-cm² flasks (Falcon Plastics) or 490-cm³ roller bottles (Corning) as previously described (3, 4). Cells were depleted of thyroid hormone before each experiment by culturing them for 12 to 24 h in Ham’s F-10 medium supplemented with AG-1-XS resin charcoal-treated calf serum (10%, v/v) which has been shown to be depleted of thyroid hormone (19). This medium was replaced with serum-free Ham’s F-10 with or without the indicated concentrations of n-butyrate or propionate for 24 h prior to the addition of 0.5 nM L-T3. This concentration of hormone results in maximal growth hormone and prolactin synthesis and serum-free growth hormone stimulation. Each day the medium from one set of cells was sampled and the cells were washed twice with 0.14 M NaCl at 4°C and frozen for later determination of cell protein (20). In the remaining cultures, the medium was exchanged, the concentration of carboxylic acid and/or L-T3 was maintained, and the incubation continued. Growth hormone and prolactin synthesis were estimated from the accumulation of growth hormone and prolactin in the culture medium over 24 h periods by radioimmunoassay as described (1, 3, 4). This provides an estimate of the average 24-h synthetic rate since GH1 cells rapidly release the synthesized peptides into the medium and growth hormone and prolactin are almost identical. These results are in good agreement with our previous observations (where only one concentration of hormone was used) and with other groups (15). Hormone synthesis in medium supplemented with 10% serum that contains hormone-binding proteins (2) was measured using 3H-L-T3 and 125I-L-T3. Details of the experimental procedure are given in the legend to Table 1.

**Analysis of Prolactin mRNA and Growth Hormone mRNA Abundance**—GH1 cells, grown in 490-cm³ roller bottles, were incubated with serum-free Ham’s F-10 medium with 0.5 mM n-butyrate for 24 h while parallel control cell cultures received no additions. At 24 h, half of the control and n-butyrate-incubated cells received 0.5 mM L-T3. The other halves were refed medium containing the same component and hormone. At 48 h, the cells were harvested with a rubber policeman into 0.14 M NaCl, 1% sodium dodecyl sulfate, 5 mM EDTA, 50 mM Tris, pH 8.3 at 25°C, with 600 μg/ml Proteinase K (24). The cell lysate was sheared by several passages through a 25-gauge needle and an aliquot was saved for DNA determination (25). Following DNase I digestion (26), phenolchloroform extraction, and ethanol precipitation, the poly(A)+ RNA was isolated by two successive oligo(dT)-cellulose chromatographic steps (27). Hybridization of amounts of poly(A)+ RNA (67 μg) from each sample were electrophoresed in 1% agarose gels containing 1% methyl mercury hydroxide (28) and blotted to DMB paper (Schleicher and Schuell) (29). The blot was then prehybridized and hybridized (30–32) to 32P-labeled pPRL-2 plasmid which had been nick translated (33) to a specific activity of 4 × 10^6 cpm/μg of DNA. The paper was then washed as described (30) and exposed at −80°C with Kodak X-Omat film using a Lightning Plus intensifying screen (Cronex). The pPRL-2 probe was then eluted with 100% formamide at 45°C and the blot was rehybridized to 32P-labeled pHt-2 plasmid (3–4 × 10^5 cpm/μg of DNA) to identify growth hormone mRNA sequences. EcoRI and PstI-BamHI digestion fragments of pBR322 were electrophoresed as standards.

**Preparation of High Molecular Weight DNA and Restriction Endonuclease Digestion**—GH1 cell cultures, incubated as described for mRNA isolation, were harvested at 4°C with a rubber policeman in 5 ml of 0.25 M sucrose, 20 mM Tris, pH 7.85 at 25°C, and 1 M MgCl2 and homogenized gently in a Teflon-glass homogenizer for 15 strokes at 500 rpm. After centrifugation, the nuclear pellet was washed in the same buffer containing 0.2% Triton X-100. The nuclei were then suspended in 5 ml of 0.25 M EDTA, 0.5% sarcosyl, and 1.5 mM EDTA (pH 8) containing n-butyrate and Triton X-100. This incubation was continued an additional 72 h at 37°C after the addition of 500 μg/sample of Proteinase K, and at 50°C for 2 h after the addition of another 500 μg of Proteinase K. The chromatin was then extracted three times with phenol (containing 0.1% 8-OH quinoline, w/v), chloroform:isoamyl alcohol (25:24:1) and dialyzed extensively at 25°C against 10 mM Tris, pH 7.5, and 1 mM EDTA. Restriction enzyme digestion was performed as described by New England Biolabs and the DNA samples were electrophoresed in 1% agarose gels (28). The gels were blotted to nitrocellulose (Schleicher and Schuell) and hybridized as described above. EcoRI and PstI-BamHI restriction fragments of pBR322 and a HindIII digest of phage were electrophoresed as standards.

**Influence of n-Butyrate and Propionate on Thyroid Hormone Nuclear Receptor Levels**—Experiments to estimate thyroid hormone nuclear receptor were performed by culturing cells in 25-cm² flasks with F-10 medium containing 10% (v/v) hormone-free calf serum for 24 h. The cells were then incubated with 5 nM L-[3,4-14]T3 for 1.5 h at 37°C and the cells were harvested and nuclei prepared as previously described (14). The radioactivity in the nuclear pellet was quantitated using a refrigerated Packard γ spectrometer at 55% efficiency followed by DNA determination (25). Nonspecific binding of L-[3,4-14]T3 to cell nuclei was estimated using a 1000-fold molar excess of nonradioactive L-T3. This value, always less than 5% of total binding, was subtracted from results obtained using radioactive hormone alone.

**Statistical Analysis**—Wherever appropriate, statistical analysis was performed using Student’s t test.

**RESULTS**

**Effect of n-Butyrate and Propionate on Thyroid Hormone Nuclear Receptor Levels**—Fig. 1 shows the effect of a 24-h incubation of n-butyrate (0.05 to 10 mM) and propionate (0.5 to 25 mM) on the level of GH1 cell thyroid hormone nuclear receptors. Both compounds decreased receptor levels with a maximal receptor reduction of 82.5% for n-butyrate and 72.5% for propionate. Both also elicited a slight increase in receptor levels at low concentrations. Propionate is approximately 5- to 10-fold less effective than n-butyrate in eliciting receptor depletion although the shapes of the response curves are almost identical. These results are in good agreement with our previous observations (where only one concentration of propionate was examined) that propionate is approximately 5- to 10-fold less effective than n-butyrate in eliciting receptor reduction and histone acetylation (14). Receptor reduction occurs without inhibition of protein synthesis by n-butyrate (14) or propionate (data not shown).
was then assessed with a 1.5-h incubation with 5 nM L-[3H]T₃ as described under "Experimental Procedures." Receptor levels in the control cells which did not receive carboxylic acid were 100 fmol/100 µg of DNA in the propionate experiment. For graphic comparison, receptor levels in each experiment are expressed as a percentage of the control value. Each point represents the average of duplicate flasks each of which varied less than 5% from the mean.

**Fig. 1.** Modulation of thyroid hormone nuclear receptor levels by n-butyrate and propionate. In separate experiments, GH₁ cells were incubated with the concentrations of n-butyrate (O) or propionate (△) indicated for 24 h. The levels of nuclear receptor were then assessed with a 1.5-h incubation with 5 nM L-[3H]T₃ as described under "Experimental Procedures." Receptor levels in the control cells which did not receive carboxylic acid were 100 fmol/100 µg of DNA in the n-butyrate study and 145 fmol/100 µg of DNA in the propionate experiment. For graphic comparison, receptor levels in each experiment are expressed as a percentage of the control value. Each point represents the average of duplicate flasks each of which varied less than 5% from the mean.

**Fig. 2.** Stimulation of growth hormone synthesis by L-T₃: influence of n-butyrate and propionate. GH₁ cells were incubated with the concentrations of carboxylic acid indicated for 24 h while one set of cells which received no additions served as a control. The medium was then replaced with medium containing the same carboxylic acid concentration with or without 0.5 nM L-T₃ as indicated. After another medium exchange at 48 h, the cells were incubated for an additional 24 h. Growth hormone production rates were determined by assaying the growth hormone which accumulated in the medium during the last 24-h period using radioimmunassay. The results were normalized per 100 µg of cell protein and are expressed as a percentage of the control cells which did not receive either L-T₃ or carboxylic acid. Growth hormone production in the control cells was 250 ng/100 µg of cell protein/24 h. Each point represents the average of triplicate cell cultures and each culture varied less than ±10% from the mean.

**Effect of n-Butyrate and Propionate on Growth Hormone Production**—Fig. 2 demonstrates that 5 to 10 mM n-butyrate, which substantially lowers receptor levels, reduces the L-T₃-induced growth hormone response approximately 80% compared to cells incubated with only hormone. However, lower concentrations of n-butyrate (0.5 and 1 mM), which can cause substantial posttranscriptional modification of chromatin proteins (11), resulted in a 36 and 26% increase in the growth hormone response to L-T₃. This increased response occurred at lower concentrations which either had no effect on receptor levels (0.5 mM) or partially reduced receptor abundance (1 mM). Growth hormone production with L-T₃ + n-butyrate at 0.5–10 mM are each significantly different than the control flasks which received only L-T₃ (p < 0.001). Without L-T₃, high concentrations of n-butyrate also decreased the basal rate of growth hormone synthesis which at 10 mM was 65% of the control cells which received no n-butyrate. Without hormone, n-butyrate did not stimulate an increase in growth hormone production at any concentration.

Fig. 2 also shows an identical experiment performed with propionate. Sufficiently high concentrations of propionate could not be achieved (due to its solubility) in hormone-treated cells to elicit a complete inhibition in growth hormone production. However, as with n-butyrate, propionate alone stimulated no increase in growth hormone synthesis while L-T₃ with 2.5 and 5 mM propionate resulted in 22 and 41% increases compared to L-T₃ alone (p < 0.0001). The propionate dose-response curve was shifted 7.5-fold rightward of the n-butyrate response curve.

**Effect of n-Butyrate and Propionate on Prolactin Production**—Fig. 3 shows the results of an experiment which examined prolactin production in control and L-T₃ (0.5 mM) cultured cells as a function of carboxylic acid concentration. In contrast with the growth hormone response, n-butyrate alone stimulated prolactin production 7.5-fold at 0.5 mM and a 2-fold stimulation was evident even at the lowest concentration examined (0.1 mM). At the highest concentration of n-butyrate (10 mM), prolactin production was inhibited 50% compared to control cells which received no additions. In addition, L-T₃ also stimulated an increase of prolactin synthesis when incubated with n-butyrate. Compared to control cells, this effect was 3-fold at the lowest concentration of n-butyrate examined (0.1 mM) and was maximal at 1 mM n-butyrate (19-fold). The L-T₃ stimulation of prolactin production decreased at high concentrations of n-butyrate over the same range at which this compound elicits a marked reduction in receptor levels (Fig. 1). Modest increases in prolactin production were observed at 0.1 to 0.5 mM propionate (1.5- to 2-fold) in cells incubated with or without L-T₃. The maximal effect of propionate was shifted 5-fold rightward of the n-butyrate response and was observed at 5 mM with and 2.5 mM without L-T₃.

**Kinetics of n-Butyrate-induced Alterations in Growth Hormone and Prolactin Synthesis**—After 24 h with 0.5 mM L-T₃ + 0.75 mM n-butyrate, growth hormone synthesis was 3-fold greater than control or n-butyrate-treated cells but was not significantly different from cells incubated with L-T₃ alone (Fig. 4A). At 48 h, L-T₃ + n-butyrate results in a 1.5-fold greater growth hormone response than cells incubated with hormone alone (p < 0.001). At 72 h, cells incubated with n-butyrate + L-T₃ synthesize 3-fold more growth hormone than the control or n-butyrate-incubated cells and almost twice that of cells incubated with only thyroid hormone (p < 0.001). Prolactin synthesis stimulated by L-T₃ + n-butyrate also showed a lag in the response but was 36-fold at 48 h and
The growth hormone production rate of the control cells was 260 ng/100 μg of cell protein/24 h while prolactin production was 1.7 ng/100 μg of cell protein/24 h. Each point represents the average of triplicate cell cultures and each culture varied less than ±10% from the mean.

**Effect of n-Butyrate on cAMP Levels—**n-Butyrate has been reported to increase cAMP levels in certain cell types (34). Since increased cAMP levels have been linked to stimulation of prolactin gene transcription (35), we examined the effect of 0.75 mM n-butyrate on basal and L-T₃-induced levels of both prolactin synthesis and intracellular cAMP. Fig. 5A shows the increased prolactin production elicited by 0.75 mM n-butyrate and 0.5 mM L-T₃ over a 48-h period. These results are essentially identical to those of Fig. 4B except that the induction by L-T₃ + n-butyrate at 48 h was 19-fold greater than the control cell cultures. In addition, the prolactin response to n-butyrate alone was slightly greater at 48 h than at 24 h. This experiment also illustrates a 50% inhibition of prolactin synthesis at 48 h by L-T₃ (p < 0.001). Fig. 5B shows no relationship between intracellular levels of cAMP and prolactin production. At 24 h, prolactin synthesis in L-T₃-treated cells declined 50% compared to control levels while the cAMP levels of these groups are identical. L-T₃ + n-butyrate induced a 2.5-fold increase in prolactin production over n-butyrate-treated cultures. Although n-butyrate increased cAMP levels approximately 25–30% over control and L-T₃-incubated cells, no significant difference between n-butyrate and L-T₃ + n-butyrate-treated cells was observed.

**Synthesis of Growth Hormone and Prolactin in GH₁ Cells Incubated with Forskolin—**To further explore whether the L-T₃ stimulation of prolactin production is mediated by cAMP, a study was performed using forskolin which stimulates cAMP by directly activating the catalytic subunit of the adenylate cyclase system (36). GH₁ cells were incubated with 10 μM forskolin and/or L-T₃, and the medium obtained between 24 and 48 h of incubation was assayed for growth hormone and prolactin (Table I). This concentration of forskolin stimulates cAMP levels 400-fold but does not stimulate growth hormone or prolactin or allow for stimulation of prolactin synthesis by L-T₃.

**Effect of n-Butyrate on Growth Hormone and Prolactin mRNA Levels—**The increased production of these polypeptide hormones could result from carboxylic acid effects to stimulate mRNA accumulation or from effects to selectively increase rates of translation. To clarify this, we performed experiments in which prolactin mRNA and growth hormone mRNA levels were examined. Fig. 6 shows the results of a typical experiment in which duplicate cell cultures were incubated with 0.75 mM n-butyrate and/or 0.5 mM L-T₃ for 48 h after a 24-h preincubation with n-butyrate. Control cells received no additions. Total cell poly(A)⁺ RNA was isolated, electrophoresed, blotted to DMB paper, and hybridized to ³²P-labeled pPRL-2 plasmid containing a cDNA to prolactin.
The identical composition at 48 h, the cells were harvested 72 h after prepared from whole cell lysates and 67 the beginning of the experiment. Total poly(A)+ RNA was then

mRNA

The gel was blotted to

Cytoplasmic prolactin mRNA

blot, the 32P-labeled pPRL-2 probe was eluted with 100% formamide

pBR322.

at about 1 kb as indicated by the arrow. Standards electrophoresed in 1% agarose gels containing 1% methylmercury hydroxide.

prolactin by radioimmunoassay and normalized per 100

Incubation Growth RNA was electropho-

The growth hormone mRNA response to n-butyrate is shown in Fig. 6B and represents the same blot as Fig. 6A which was hybridized to a probe for growth hormone mRNA (32P-labeled pRGH-1) after elution of the labeled pPRL-2 probe. Control cell cultures (lanes 1 and 2) show a 1-kb species corresponding to growth hormone mRNA (16). As estimated by densitometry, L-T3 (0.5 nM) (lanes 3 and 4) stimulated a 5- to 10-fold increase in growth hormone mRNA. Lanes 5 and 6 show results with 0.75 mM n-butyrate alone which are essentially identical to the control cells. L-T3 + n-butyrate (lanes 7 and 8) stimulated an increase in growth hormone mRNA abundance which was 5-fold greater than L-T3 alone.

As with prolactin, the growth hormone production rates paralleled growth hormone mRNA abundance for each of the incubation conditions (Table II).

Growth Hormone and Prolactin Gene Methylation in GH3 Cells—Since the studies of Bird and Southern (37), it has been shown that many actively transcribed genes are under- methylated compared with those that are transcriptionally dormant (38). Recent studies have shown that n-butyrate incubation can decrease DNA methylation in Friend murine erythroleukemia cells (38). This suggested that the effect of n-butyrate and/or L-T3 might involve a mechanism which results in undermethylation of the growth hormone and prolactin genes in GH3 cells. GH3 cell DNA was isolated and incubated with several restriction endonucleases whose specificity with regard to methylated sequences are known (18). HpaII and MspI are isoschisomers which cleave the sequence CCGG. However, HpaII will not cleave this sequence if the second cytosine is methylated (49). MspI cleaves both the methylated and unmethylated sequence. Aval cleaves the sequence C(T or C)CGA or GIA but will not digest the sequence if the cytosine preceding the guanine is methylated (37).

FIG. 6. Effect of n-butyrate on prolactin and growth hor- mone mRNA levels. GH3 cells were incubated with 0.75 mM n-butyrate for 24 h while parallel control cell cultures received no additions. At 24 h, half of the control and n-butyrate-incubated cells received 0.5 nM L-T3. After exchanging the medium for medium with the identical composition at 48 h, the cells were harvested 72 h after the beginning of the experiment. Total poly(A)+ RNA was then prepared from whole cell lysates and 67 μg of RNA was electrophoresed in 1% agarose gels containing 1% methylmercury hydroxide. The gel was blotted to DMB paper, hybridized with 32P-labeled pPRL-2 plasmid, and autoradiographed using an intensifying screen at -80°C. Lanes 1 and 2, control cells; lanes 3 and 4, 0.5 nM L-T3; lanes 5 and 6, 0.75 mM n-butyrate; and lanes 7 and 8, L-T3 + n-butyrate.

Cytoplasmic prolactin mRNA (A) electrophoreses as a 1-kb species while nuclear precursors migrate as 1.7-1.8 kb. After exposing the blot, the 32P-labeled pPRL-2 probe was eluted with 100% formamide at 45°C and the blot was rehybridized with labeled pRGH-1 plasmid.

Cytoplasmic growth hormone mRNA (B) migrates as a single species at about 1 kb as indicated by the arrow. Standards electrophoresed in a parallel lane consisted of a PvuII-BstNI and an EcoRI digest of pBR322.

TABLE II

Growth hormone and prolactin production with L-T3 and n-butyrate

| Incubation conditions | Growth hormone | Prolactin |
|-----------------------|----------------|-----------|
|                       | pg/100 μg DNA | pg/100 μg DNA |
| Control               | 293           | 5         |
| n-Butyrate (0.75 mM)  | 361           | 35        |
| L-T3 (0.5 nM)         | 5,610         | 4         |
| L-T3 + n-butyrate     | 39,500        | 1,021     |

mRNA (Fig. 6A). Control cells (lanes 1 and 2) show a distinct 1-kb species which represents mature cytoplasmic prolactin mRNA. This was slightly decreased in L-T3-cultured cells (lanes 3 and 4). The cells incubated with n-butyrate alone (lanes 5 and 6) show increased prolactin mRNA levels while those cultured with both L-T3 and n-butyrate (lanes 7 and 8) show a striking increase in prolactin mRNA abundance. As estimated by densitometry, the intensity of the autoradiographic bands parallels the amount of prolactin produced during the 24-h period preceding mRNA isolation (Table II). In addition, a 1.7-1.8-kb nuclear precursor of prolactin mRNA was observed when cells are incubated with L-T3 and n-butyrate (lanes 7 and 8). A 1.7-1.8-kb species has been reported to be a highly abundant processed form of the prolactin gene transcript in GH3 cells (24).

The growth hormone mRNA response to n-butyrate is shown in Fig. 6B and represents the same blot as Fig. 6A which was hybridized to a probe for growth hormone mRNA (32P-labeled pRGH-1) after elution of the labeled pPRL-2 probe. Control cell cultures (lanes 1 and 2) show a 1-kb species corresponding to growth hormone mRNA (16). As estimated by densitometry, L-T3 (0.5 nM) (lanes 3 and 4) stimulated a 5- to 10-fold increase in growth hormone mRNA. Lanes 5 and 6 show results with 0.75 mM n-butyrate alone which are essentially identical to the control cells. L-T3 + n-butyrate (lanes 7 and 8) stimulated an increase in growth hormone mRNA abundance which was 5-fold greater than L-T3 alone.

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Fig. 7 (left) shows an autoradiograph from an MspI digest of GH3 cell DNA electrophoresed in a 1% agarose gel and hybridized against 32P-labeled pRGH-1. Based on the restriction map of the growth hormone gene cloned from rat liver DNA, MspI or HpaII should yield DNA fragments of 0.4 and 0.5 kb if the DNA restriction sequence is unmethylated (41, 42). If the sequence is methylated, HpaII will not generate these fragments but the 0.4- and 0.5-kb fragments will be excised by MspI. HpaII digestion of GH3 cell DNA yielded hybridizable fragments of 9.2 and 6.3 kb (not illustrated) while MspI generated fragments of 4.9, 4.3, 0.5, and 0.4 kb. These results indicate that the HpaII-MspI restriction sites within the growth hormone gene are methylated. The identification of the 4.9 and 4.3 MspI and the 9.2- and 6.3-kb HpaII restriction fragments suggest that one or more HpaII-MspI restriction sites flank the growth hormone gene. These sites appear to be heterogeneously methylated as indicated by the different restriction fragments excised by MspI and HpaII.
Fig. 7. Restriction endonuclease analysis of the GH₁ cell growth hormone gene. The experimental protocol was identical to that described in Fig. 6 and the cells were harvested after 72 h of incubation. High molecular weight DNA was then prepared from these cells as described under "Experimental Procedures" and the DNA was digested with *Msp*I (left) or *Hind*III and *Aval*I (right). Hybridization was performed using nick-translated ³²P-labeled pRGH-1 plasmid containing growth hormone gene sequences. Lanes are marked from left to right: control, 0.5 mM L-T₃, 0.75 mM n-butyrate, and 0.75 mM n-butyrate + 0.5 mM L-T₃. *Eco*RI and *Pst*I-*Rsa*I restriction fragments of pBR322 and a *Hind*III digest of λ phage were electrophoresed in parallel lanes as standards.

Fig. 8. Restriction endonuclease analysis of the GH₁ cell prolactin gene. After exposing the blot from Fig. 7, the plasmid probe was eluted at 65 °C with several changes of 5 mM Tris, pH 7.9, 0.2 mM EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinylpyrrolidone, 0.002% Ficoll, and 0.002% bovine serum albumin by the method of Thomas (54). The blot was then rehybridized to ³²P-labeled pPRL-2 plasmid. The *Msp*I and the *Hind*III-*Aval*I digests and the lane designations are as in Fig. 7.

of the predicted doublet fragments is too small to be resolved in this gel.

The blots hybridized to ³²P-labeled pRGH-1 in Fig. 7 were also hybridized against labeled pPRL-2 (Fig. 8). Fig. 8 (left) shows the results of *Msp*I digestion of GH₁ DNA while Fig. 8 (right) shows the *Hind*III-*Aval*I digest. If the prolactin gene is unmethylated at the recognition site for HpaII-*Msp*I, both enzymes will yield fragments of 1.7 and 4.3 kb (43). However, if this site is methylated, only *Msp*I will yield these fragments. Since bands of 1.7, 3.9, and about 6.0 kb are generated by *Msp*I and no fragments were identified from a HpaII digest (data not shown), the three known HpaII-*Msp*I sites in the prolactin gene are methylated. It also appears, from identification of the 6.0-kb fragment, that one other site probably exists flanking the prolactin gene that is also methylated. Alternatively, the 6.0-kb fragment may reflect an incomplete digestion to yield the 1.7- and 3.9-kb restriction fragments.

Simultaneous digestion of the prolactin gene using *Hind*III and *Aval*I would be expected to yield two fragments of 5.9 and 5.1 kb if the *Aval*I site in the prolactin gene is methylated but 5.1-, 3.1-, and 2.8-kb species if unmethylated (43). The blot in Fig. 8 (right) identified fragments estimated as 4.9, 4.7, 3.4, and 3.0 kb. The two larger species are in an area of the gel where molecular weight determinations are not precise while the smaller fragments are within 10% of their expected size. Therefore, the *Aval*I sites in the prolactin gene appear to be variably methylated. Of significance, no major difference in the methylation of the prolactin or the growth hormone genes in response to either L-T₃ and/or n-butyrate was apparent by restriction enzyme analysis in Figs. 7 and 8.

**DISCUSSION**

In this study, we have examined the relationship between carboxylic acid alteration of thyroid hormone nuclear receptor levels and the L-T₃ regulation of growth hormone and prolactin production. Fig. 1 shows that n-butyrate elicits a biphasic effect on thyroid hormone receptor levels with low concentrations (0.05–0.25 mM) stimulating a 20 to 30% increase and higher concentrations (>0.5 mM) decreasing receptor abundance. Propionate exhibited the same biphasic receptor response but was shifted 10-fold rightward of the n-butyrate response. Fig. 2 illustrates that n-butyrate or propionate alone did not stimulate growth hormone production at any concentration examined. With L-T₃, however, n-butyrate between 0.5 and 1 mM resulted in a 25 to 30% stimulation of growth hormone production compared to L-T₃ alone. With higher n-butyrate concentrations, L-T₃ stimulation of growth hormone synthesis decreased in parallel with the n-butyrate concentration-dependent decrease in receptor levels (Fig. 1). Stimulation of growth hormone synthesis by L-T₃ was also influenced by propionate except that the response was shifted approximately 10-fold rightward of the n-butyrate-mediated effect.

n-Butyrate and propionate incubation can elicit a paradoxical prolactin response to L-T₃ (Fig. 3). In contrast with the growth hormone response, n-butyrate alone (0.5 mM) increased the basal prolactin production 7.5-fold with a decrease in the response occurring at higher n-butyrate concentrations (Fig. 3). Surprisingly, prolactin production and mRNA levels, which are modestly inhibited by L-T₃, were stimulated about 20- to 70-fold by L-T₃ + n-butyrate (0.5–1 mM) (Figs. 3 and 6; Table II). A similar response to propionate or L-T₃ + propionate was also observed except that the response was again shifted 5- to 10-fold rightward of the n-butyrate effect.

Stimulation of each response by L-T₃ + n-butyrate (0.75 mM) is secondary to an increase in the accumulation of prolactin and growth hormone mRNA (Fig. 6). Since growth hormone mRNA has an estimated half-life of 50 h (7), it is unlikely that further prolongation of the half-life could account for the extent of growth hormone mRNA accumulation observed in Fig. 6. In addition, a 1.7–1.8-kb nuclear precursor of prolactin mRNA (24) was identified in GH₁ cells incubated with L-T₃ + n-butyrate (Fig. 6). These observations suggest that the L-T₃ + n-butyrate stimulation of prolactin and growth hormone synthesis is secondary to mRNA accumulation and likely reflects mRNA synthesis rather than prolongation of mRNA half-life.

The kinetics of stimulation of growth hormone and prolac-
tion and globin synthesis in Friend erythroleukemic cells (39), HpaII-MspI restriction sites in both the growth hormone and prolactin genes are methylated and the we examined whether n-butyrate incubation resulted in hy-
proteins.

butyrate incubation results in DNA hypomethyla-
tion and globin synthesis in Friend erythroleukemic cells (39), we examined whether n-butyrate incubation resulted in hy-
proteins.
imethylation of growth hormone and prolactin gene se-
quencies in GH1 cells. Figs. 7 and 8 demonstrate that the
Hpall-MspI restriction sites in both the growth hormone and prolactin genes are methylated and the Acal site in both genes is unmethylated. We are not aware of a previous study which examined prolactin gene methylation in relation to stimula-
tion or inhibition of the prolactin response. However, our findings for the growth hormone gene are in agreement with
those of Moore et al. (47). L-T3 or n-butyrate ± L-T3 induced
no alteration in the extent of methylation of prolactin or growth hormone gene sequences based on this analysis. These studies exclude global but not site-specific effects of n-buty-
rate on methylation of these genes since our analysis is limited to
those sequences which allow for isoschisomer restriction enzyme analysis.

Since Murdoch et al. (35) reported that forskolin, which markedly elevates CAMP levels, stimulates prolactin gene transcription and mRNA accumulation in GH1 cells, we ex-
amined the long term effect of forskolin on the prolactin response in GH1 cells. These studies (Table I) show that forskolin stimulated a 400-fold increase in CAMP production but had no effect on basal prolactin production and did not allow for stimulation of prolactin synthesis by L-T3. In the study by Murdoch et al. (35), the effect of CAMP was attrib-
uted to the phosphorylation of a M, = 23,000 basic nonhistone protein and it is possible that this regulatory protein is absent
from GH1 cells.

n-Butyrate enhancement of the hormonal stimulation of
certain proteins (e.g. alkaline phosphatase) may be secondary
to a G1 arrest where hormonal regulation may be more pro-
ounced than at other stages of the cell cycle (44). However, it is unlikely that the marked stimulation of prolactin mRNA by l-T3 + n-butyrate (Fig. 6) is explained by arrest in the G1 phase. First, the concentrations of n-butyrate used to elicit the L-T3 response (0.75 mM) is lower than that (≥2.5 mM) shown to arrest other cell types in G1 (44), and we have previously shown that 0.75 mM n-butyrate does not alter cell growth or the DNA content per culture compared to control cells (14). Furthermore, 6 mM propionate, a concentration at which maximal L-T3 stimulation of growth hormone and prolactin occurs (Figs. 2 and 3), has been reported not to inhibit cell growth (13). Second, without n-butyrate, at least 40% of growing cells would be expected to be in the G1 period (48). Therefore, asynchronous cells cultured without n-butyrate
but with L-T3 should show some stimulation of prolactin synthesis or mRNA accumulation. Since L-T3 decreases pro-
lactin production and mRNA levels (Figs. 5 and 6), an n-
butyrate increase in the fraction of cells in the G1 phase does not provide an explanation for the paradoxical regulation of the prolactin response by L-T3.

Kimura et al. (49) recently reported that inhibition of ADP-
ribosylation in GH3 cells resulted in a 3- to 4-fold increase in basal growth hormone and prolactin production and also further enhanced the L-T3 induction of growth hormone syn-
thesis. However, inhibition of ADP-ribosylation did not result in stimulation of prolactin synthesis by L-T3, as observed with n-butyrate in our studies. Stimulation of phosphorylation of chromatin-associated proteins and histone methylation has not been examined in GH3 or GH4 cells but have been reported to occur in other cell types using concentrations of n-butyrate of 5 mM or higher (45). Although our studies do not exclude these postsynthetic modification events, n-butyrate concen-
trations greater than 5 mM decrease rather than enhance the effect of L-T3 on the growth hormone and prolactin response.

The acetylation of core histones and other nuclear proteins is the most extensively examined effect of n-butyrate in cells (50). First demonstrated by Riggs et al. (10) in HeLa cells and in erythroleukemic cells, this effect has been shown to occur in a wide variety of cell lines (50). The effects of n-butyrate and propionate described in this paper are consistent with a mechanism which involves increased acetylation of histones or other chromosomal proteins. First, the 5- to 10-fold shift in the n-butyrate and propionate dose-response curves (Figs. 1-3) is in agreement with the potency of these compounds in
stimulating acetylation as a consequence of their relative inhibitory effects on chromatin-associated deacetylase activ-
ity (12). Furthermore, the long n-butyrate incubation period required for prolactin stimulation by L-T3 is also consistent with an effect on the acetylation process. Although a large fraction of histones is deacetylated with a t50 of minutes,
acetylation of a subset of the histone population only occurs after very long n-butyrate incubation times (13).

Although our studies suggest a relationship between inhibi-
tion of chromatin-associated deacetylase activity and L-T3 stimulation of prolactin and growth hormone mRNA produc-
tion, the precise mechanism surrounding these events remains unclear. Analysis of n-butyrate effects in cells indicates that the carboxylic acid only stimulates or inhibits the expression of a small fraction of the genome (51). Of considerable interest is that low concentrations of n-butyrate reverse the prolactin response to L-T3. In most experiments, L-T3 modestly inhibits the prolactin response 25 to 50%. n-Butyrate alone moder-
ately stimulates the prolactin response and with n-butyrate thyroid hormone acts as a potent stimulator of prolactin
synthesis and mRNA production (Figs. 3-6; Table II). This suggests that prolactin gene expression is under partial regu-
lation by a regulator which is reversed as a consequence of n-
butyrate-mediated postsynthetic modification events. This
could reflect an alteration of chromatin structure, or postsyn-
thetic modification and inactivation of a regulatory regu-

lar factor(s) of prolactin gene expression.

The growth hormone and prolactin genes are closely related and are presumed to have arisen from gene duplication or other recombinant events (52). Both genes share many com-
mon sequences and show some sequence homology in the 5' region flanking the structural gene where hormone-receptor control of genes has been shown to occur (53). Therefore, both growth hormone and prolactin genes may contain similar thyroid hormone receptor promoter sequences in the 5'-flanking regions. A working model to explain our results is that repression of prolactin gene expression is reversed by n-
butyrate incubation which allows for stimulation of gene expression by the thyroid hormone-receptor complex. This is observed at low concentrations of n-butyrate where no de-
crease in thyroid hormone receptor occurs. Within this frame-
work, the modest L-T3 inhibition of prolactin production could
occur by direct or indirect enhancement of a putative repressor component(s). Although this model is speculative it can be tested by appropriate transfection studies with a recombinant fusion gene containing the 5′-flanking region of the prolactin gene.

Acknowledgment—We would like to thank Mary McCarthy for expert secretarial assistance.

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n-Butyrate effects thyroid hormone stimulation of prolactin production and mRNA levels in GH1 cells.
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J. Biol. Chem. 1984, 259:9768-9775.

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