THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 30, Issue of October 25, pp. 13997-14004, 1986
Printed in U.S.A.

Modulation of Thyroid Hormone Nuclear Receptors by Short-chain Fatty Acids in Glial C6 Cells

ROLE OF HISTONE ACETYLATION*

(Received for publication, March 5, 1986)

Javier Ortiz-Caro, Fátima Montiel, Angel Pascual, and Ana Aranda

From the Departamento de Endocrinología Experimental, Instituto de Investigaciones Biomedicas, Consejo Superior de Investigaciones Científicas and Facultad de Medicina, Universidad Autonoma de Madrid, 28029 Madrid, Spain

We have studied the effect of butyrate and other short-chain fatty acids on thyroid hormone nuclear receptors in C6 cells, a rat glioma cell line. Exposure of C6 cells to butyrate leads to increased levels of L-triiodothyronine (T3) in the nuclear and extranuclear compartments. The rise in nuclear binding is not merely a reflection of the higher cellular hormone content, and Scatchard analysis of T3 binding to isolated nuclei reveals that butyrate increases receptor number without changing affinity. The effect on the receptor is quantitatively important: a 48-h incubation with 2 mM butyrate increases nuclear binding by 2–3-fold, and 5 mM butyrate by 3–5-fold. Other short-chain fatty acids were found to similarly influence both nuclear receptor and extranuclear T3 levels with the following potency: butyrate > valerate > propionate > acetate. On the contrary, ketone bodies were ineffective. Butyrate increases receptor levels by decreasing receptor degradation, since the apparent $t_{1/2}$ of receptor disappearance increased by approximately 3-fold in cells incubated with 2 mM butyrate for 48 h. The regulation of receptor number might be secondary to an action of butyrate on regions of the chromatin to which the receptor associates. We then examined the effect of butyrate on histone acetylation. The fatty acid had little effect in increasing the level of multiacetylated forms of H3 and H4 histone when studied in acid-urea gels, but it markedly inhibited the turnover of [^{3}H] acetate from the histone fraction. There was a striking similarity in the dose-response of butyrate for increasing receptor levels and inhibiting histone deacetylation. Furthermore, a very close correlation between receptor levels and [^{3}H]acetate release was also found when different short-chain fatty acids were used. We thus conclude that the effect of butyrate on the receptor could be explained by a modification of the chromatin structure of C6 cells secondary to acetylation.

Thyroid hormone actions in cells appear to be initiated by a nuclear-associated receptor, an acidic nonhistone chromatin-associated DNA-binding protein (1). The level of receptor is determined by its rate of synthesis and degradation, which in turn could be influenced by the functional state of the chromatin to which the receptor associates. Although little is known regarding which components or aspects of chromatin structure might modify the nuclear concentration of thyroid hormone receptors, it would not be surprising that postsynthetic modifications of chromatin proteins may affect the receptor.

Butyrate,[1] a naturally occurring short-chain fatty acid, produces a wide variety of effects on cells in culture (2), influencing cellular proliferation and differentiation (3–6), and altering the expression of specific genes (7–13). This compound increases the level of acetylation of core histones, mainly H3 and H4 (14–16), and other nuclear proteins (17) by inhibiting deacetylation (15, 16, 18–21). Other short-chain fatty acids produce similar effects although they are less active (16, 18, 21). In addition to the extensively studied influence on deacetylation, butyrate also has other nuclear actions including modifications in the extent of phosphorylation, methylation, or ADP-ribosylation of chromatin-associated proteins (22). Apart from its nuclear effects, butyrate could act on several cell compartments. In this respect it has been shown that this compound influences cytoskeleton assembly (23) and membrane composition (24) and function (25).

Using GH1 cells Samuels *et al.* (21) have demonstrated that butyrate elicits a reduction in the nuclear thyroid hormone receptor levels which is inversely related to the extent of histone acetylation. Using a photoaffinity label probe, it has been shown that in GH1 cells the receptor has two different molecular weight forms, an abundant 47,000 M_r form and a minor 57,000 M_r form (26) and that butyrate decreases receptor levels primarily by shortening the half-life of the 47,000 M_r form (27). In addition, at low concentrations of butyrate (0.5 mM or less) a small increase in receptor number is observed (28), which is due to an increase of the 57,000 M_r form (27).

C6 cells, a rat glioma cell line, possess thyroid hormone receptors (29) and are a very valuable model for normal glia in a number of respects (30) since their responsiveness to several hormones closely resembles that of nontransformed glial cells in culture (13).

In the present study we have examined the regulation of the receptor by butyrate in glial C6 cells. Our results indicate that butyrate, and other short-chain fatty acids, elicit a time- and dose-dependent increase of the thyroid hormone nuclear receptor in these cells. This effect is reversible and appears to be mediated by an increase in the receptor half-life. The action of butyrate is probably related to its influence in deacetylation of nuclear proteins, since the dose-dependence and the potency of the different analogs was very similar for both effects. In addition, butyrate also increased extranuclear

* This research was supported by research grants from the Comisión Asesora de Investigación Científica y Técnica and from the Fondo de Investigationes Sanitarias de la Seguridad Social of Spain. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: butyrate, sodium butyrate; T3, 3,5,3′-triiodo-L-thyronine.

hormone levels, although the effect on the nuclear receptor appears to be an independent phenomenon.

EXPERIMENTAL PROCEDURES

Hormones and Chemicals—L-3,5,3′-[^{125}I]Triiodothyronine (specific activity 1200 mCi/mg) was obtained from New England Nuclear. [^{3}H] acetic acid, sodium salt (specific activity 2.9 Ci/mmol) was from The Radiochemical Center (Amersham Corp.). Cell culture products were from Gibco Laboratories. Resin AG 1X-8 was obtained from Bio-Rad. All other reagents were obtained from Eastman or Sigma and were of the highest purity available.

C6 Cell Culture—C6 cells were grown in monolayer cultures. The cells were inoculated at initial densities of 30,000–60,000 cells/cm^2 with RPMI 1640 medium containing 2.5% fetal calf serum and 10% horse serum and incubated for 72 h at 37 °C in an atmosphere of 95% air, 5% CO_2. The medium was replaced with RPMI medium containing 10% (v/v) newborn calf serum depleted of thyroid hormone by treatment with AG 1X-8 resin and charcoal as previously described (31). The cells were incubated with this medium for an additional 24–48 h before the beginning of the experiments to ensure the complete cellular depletion of thyroid hormone.

Quantitation of Thyroid Hormone Nuclear Receptor Levels—[^{125}I] T3-binding studies were carried out in intact cells and in isolated nuclei as described by Samuels *et al.* for pituitary GH1 cells (32, 33). C6 cell monolayers were incubated with butyrate or other compounds in thyroid hormone-depleted medium at the concentrations and for the times indicated in the text. For the binding studies in intact cells, the medium was replaced by serum-free RPMI and the cells were incubated either with increasing concentrations of [^{125}I]T3 for 3 h (for Scatchard analysis), or for 90–120 min with 0.8 nM [^{125}I]T3. In serum-free medium conditions, this concentration of T3 gives an estimate of the total receptor levels since it binds to more than 85% of the receptor population. After incubation the cells were chilled at 4 °C, the nuclei isolated, and nuclear binding and extranuclear T3 content determined as previously described (33). Extranuclear T3 was considered to be the total cell [^{125}I]T3 minus the nuclear bound hormone. Nonspecific nuclear binding was determined by incubating cells with a 1000-fold excess of nonradioactive T3. In the nuclear fraction the nonspecific binding ranged between 1 and 3 fmol/100 μg of DNA and was substracted from the total to determine the receptor-bound T3. The extranuclear fraction did not demonstrate saturable component(s). The data from the cultures receiving the excess of cold T3 were discarded for the calculation of extranuclear T3 levels. Binding assays were also carried out in isolated nuclei as described by Samuels *et al.* (32), by incubating nuclei from control cells or from butyrate-treated cells with different concentrations of [^{125}I]T3.

Extraction of Histones and Gel Electrophoresis—Nuclei were washed at 0 °C with buffer containing 0.14 M NaCl, 2.4 mM $MgCl_2$, and 5 mM Tris-HCl, pH 7.4. The histones were extracted twice with 0.4 N H_2SO_4 as described (21) and precipitated overnight at −20 °C with 10 volumes of acetone. The histones were collected by centrifugation at 37,000 × *g* for 20 min, dissolved in 0.9 N acetic acid, and electrophoresed in slab acid-urea-polyacrylamide gels at pH 2.7 with 2.5 mM urea as described by Panyim and Chalkley (34). Approximately 40–60 μg of histone were applied to each lane. After electrophoresis the gels were stained with Amido Black, destained, and dried.

Influence of Butyrate and Other Short-chain Fatty Acids on Histone Deacetylation—C6 cells from a 75-cm^2 flask were detached from the flask, transferred to tubes, washed with medium, and resuspended in 1 ml of RPMI medium containing 1 mCi ^{3}H-acetate. The cell suspensions were incubated at 37 °C for 1 h, and the pulse was terminated by chilling and centrifugation. Cell pellets were extensively washed with cold medium before resuspension in warm (37 °C) medium for subsequent chase periods. Chase was carried out for different times in the presence or absence of butyrate or other analogs at the concentration indicated in the text. After the chase period the cells were washed with ice-cold phosphate-saline buffer and the nuclei isolated as previously described (32, 33). The histones were extracted and analyzed by electrophoresis as described above. The gels were dried, photographed for posterior densitometry of the protein bands, and cut for determination of radioactivity in each histone subfraction in a β-counter after solubilization with 30% hydrogen peroxide. In other occasions the gels, after photography, were submitted to fluorography by the method of Chamberlain (35), and dried for autoradiography. Densitometry of the bands was carried out in a gel scanner (Quick Scan Jr, Helena Laboratories), and the calculation of the areas of the stained or the radioactive peaks was done with a Graphic Tablet of the Apple IIe computer. Specific activity of the bands was calculated as the ratio of the radioactivity area to the protein area of each peak.

Estimation of the $t_{½}$ of Nuclear Receptor—Receptor disappearance was estimated after inhibition of protein synthesis with cycloheximide. Triplicate cultures were preincubated with or without butyrate for 48 h. The cells were then incubated in the presence or absence of 5 μM cycloheximide, which inhibited [^{3}H]leucine incorporation into proteins by more than 95%, for different time periods, and for the last 90 min with 0.8 nM [^{125}I]T3 to estimate nuclear receptor levels.

Other Determinations—DNA was determined by the method of Burton (36) using 2-deoxyribose as a standard. Histone was measured by the method of Lowry *et al.* (37). Each data point of [^{125}I]T3 binding to C6 cells is the mean of triplicated cultures that did not vary more than ±5–10%. Data are presented as mean ± S.D.

RESULTS

Influence of Butyrate on [^{125}I]T3 Binding to C6 Cells—Fig. 1 illustrates the time-course of the butyrate effect on nuclear receptor and extranuclear T3 levels. The effect of 2 mM butyrate was examined over a 53-h period. Nuclear receptor levels (*left panel*) were 30 fmol/100 μg DNA in control cells and increased in a time-dependent fashion after exposure to butyrate. Cells incubated with butyrate showed a 3-fold increase in nuclear receptor at 53 h, and longer incubation times did not elicit a further increase. Butyrate also elicited a similar elevation of extranuclear T3 levels, although it took a longer time to be detected. Both effects were also observed when the cells were incubated with the fatty acid in serum-free medium (not illustrated), thus demonstrating that other factor(s) present in the serum are not necessary for the action of butyrate to occur.

Butyrate affected proliferation of C6 cells, because this compound decreased total DNA/culture in a time-dependent fashion. An inhibition of 30–40% was found after incubation with 2 mM butyrate for 48 h. However, DNA content per cell did not change significantly (8–10 pg/cell in both control and butyrate-treated cells). The effect of butyrate on the receptor and extranuclear T3 does not reflect a generalized increase in protein synthesis, since it did not change [^{3}H]leucine incorporation into proteins (not illustrated).

Fig. 2 shows the influence of a 24-h incubation with 2 mM butyrate on the Scatchard plot of T3 nuclear binding assessed in intact cells. The estimated K_d were similar (0.15 nM in control cells and in butyrate-treated cells) indicating that butyrate does not alter the affinity of the receptor for T3 but increases the estimated number of binding sites. The results of the Scatchard plot suggest that the increase in nuclear binding observed in the butyrate-treated cells is not merely a reflection of the higher total cellular T3 content. This was

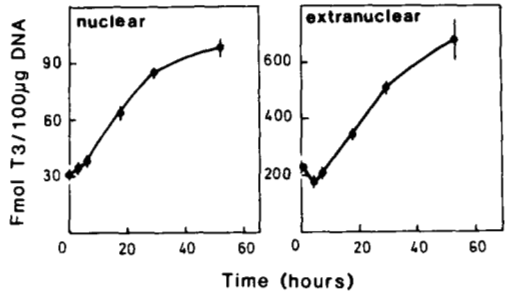

FIG. 1. **Time-course of the butyrate effect on nuclear receptor and extranuclear T3 levels.** C6 cells were incubated with 2 mM butyrate for the times indicated. The cells were then shifted from serum-containing to serum-free medium and incubated for 90 min with 0.8 nM [^{125}I]T3. T3 binding to the nuclear receptor (*left panel*) and extranuclear T3 concentration (*right panel*) were determined as described under "Experimental Procedures."

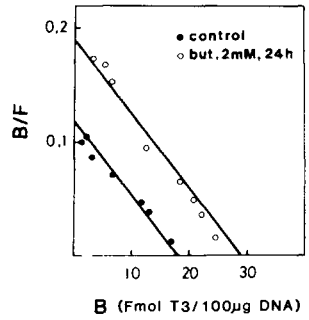

FIG. 2. **Scatchard plot of [^{125}I]T3 nuclear binding in intact cells.** C6 cells were incubated with 2 mM butyrate for 24 h. These cells along with control cells which did not receive butyrate were incubated for 3 h with different concentrations of [^{125}I]T3, and the receptor-bound [^{125}I]T3 was determined. Control cells (●); butyrate-incubated cells (○).

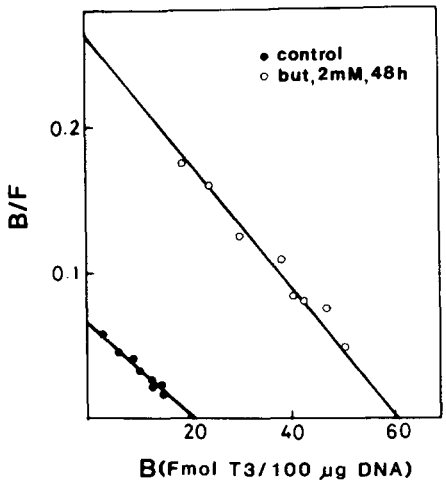

FIG. 3. **Scatchard plot of [^{125}I]T3 binding to isolated nuclei.** C6 cells were incubated with 2 mM butyrate for 48 h. Nuclei were then isolated and incubated for 3 h with different concentrations of [^{125}I]T3. Control (●); butyrate (○).

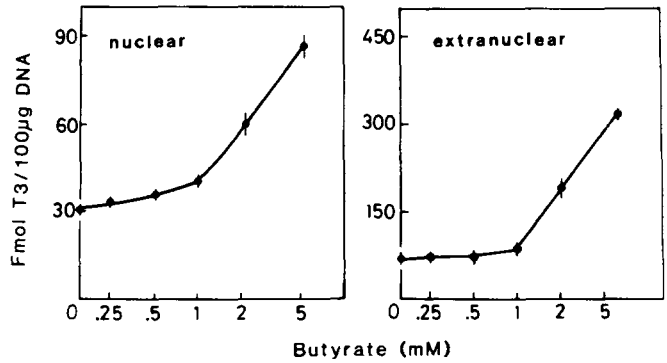

FIG. 4. **Influence of butyrate concentrations on T3 nuclear receptor and extranuclear T3 levels.** C6 cells were incubated with the concentrations of butyrate indicated for 48 h. Thyroid hormone receptor levels (*left panel*) and total extranuclear bound hormone (*right panel*) were determined after incubation with 0.8 nM [^{125}I]T3 for 90 min in serum-free medium.

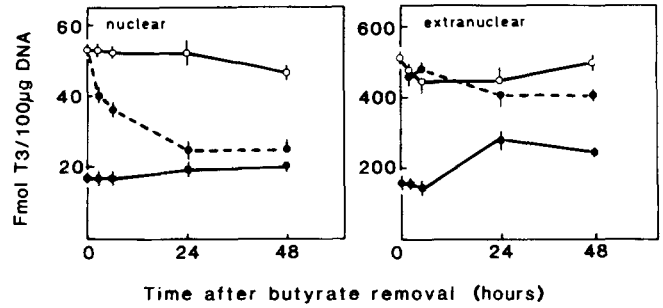

FIG. 5. **Thyroid hormone receptor decrease following butyrate removal.** The cells were preincubated with or without 2 mM butyrate for 48 h. At the end of the incubation period all cultures were washed with fresh medium (*time 0* in the figure). Half of the cultures which had been incubated with butyrate received again 2 mM butyrate, whereas the other half of the cultures received the medium alone. T3 receptor (*left panel*) and extranuclear T3 levels (*right panel*) were determined at different times between 3 and 48 h by a 90-min incubation with 0.8 nM [^{125}I]T3. Control (●——●); butyrate (○——○); butyrate-removed (●- -●).

further demonstrated by studying the binding of [^{125}I]T3 to isolated nuclei. Fig. 3 shows a Scatchard plot of T3 binding to nuclei from control cells and from cells incubated with 2 mM butyrate for 48 h. The estimated K_d values were similar (0.67 and 0.46 nM, respectively) and there was an almost 3-fold increase in the maximal binding capacity in conditions where there is no interference of extranuclear T3 levels.

Fig. 4 shows the dose-response of butyrate effect on nuclear receptor and extranuclear [^{125}I]T3 levels. C6 cells were incubated for 48 h with butyrate 0.25–5 mM. Concentrations lower than 1 mM were ineffective, but higher doses increased receptor levels in a dose-dependent manner. Concentrations higher than 5 mM did not elicit a further increase of receptor (not shown), and therefore a half-maximal effect was obtained at approximately 1.5 mM butyrate. Extranuclear T3 also increased at concentrations of butyrate higher than 1 mM.

Reversal of Butyrate Effect After Butyrate Removal—Fig. 5 shows the reversibility of receptor accumulation and extranuclear increase. C6 cells which had been cultured in the presence of 2 mM butyrate for 48 h (time 0) were either washed free of butyrate or grown in the continuous presence of the fatty acid. Receptor levels were measured in these cells as well as in groups of control cells at different times ranging between 3 and 48 h later. Receptor levels remained constant in control cells and in cells incubated with butyrate. Receptor number gradually decreased after butyrate removal and was already normal 24 h after washing the cells free of butyrate. By contrast with the receptor data, the extranuclear T3 levels were not easily reversed. Extranuclear T3 was not decreased after 24 h of butyrate removal when receptor levels were already normal and after 48 h, extranuclear T3 was 161% of the control in butyrate-washed cells and 205% in cells receiving butyrate during the whole period. DNA levels recover with a time-course similar to the extranuclear T3 and therefore more slowly than the receptor.

Effect of Butyrate on the $t_{1/2}$ of Receptor Disappearance after Inhibition of Protein Synthesis—Fig. 6 shows an estimate of the $t_{1/2}$ of receptor disappearance after incubation with cycloheximide in control cells and in cells incubated with 2 mM butyrate for 48 h. The initial rate of receptor disappearance was linear in a semilogarithmic plot and was slower in the cells incubated with butyrate. However, after longer incubation times with cycloheximide, the receptor also decreased very slowly in control cells and after 24 h less than a 50% decrease was found. This could mean that cycloheximide could not only be blocking receptor synthesis but also affecting receptor degradation. An extrapolated $t_{1/2}$ of receptor disappearance was calculated from the 6 first h and was 11–12 h in control cells and 31–32 h in the cells incubated with butyrate.

Influence of Different Short-chain Fatty Acids and of Ketone Bodies on Nuclear and Extranuclear [^{125}I]T3 Binding—A comparison of the effect of butyrate and that of other fatty

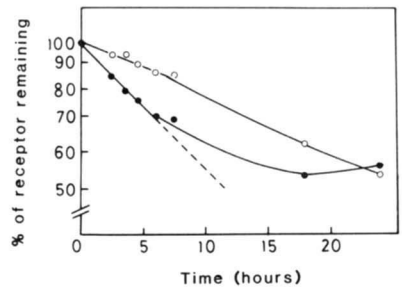

FIG. 6. **Disappearance of thyroid hormone nuclear receptor after inhibition of protein synthesis.** C6 cells were incubated in the presence or absence of 2 mM butyrate for 48 h. The cells were then incubated with 5 μM cycloheximide and nuclear receptor levels determined as described under "Experimental Procedures." At time 0 the levels of receptor were 25 and 61 fmol of [^{125}I]T3/100 μg of DNA in control and butyrate-treated cells, respectively. The data are expressed as percent of these initial values. Control cells (●); butyrate-treated cells (○).

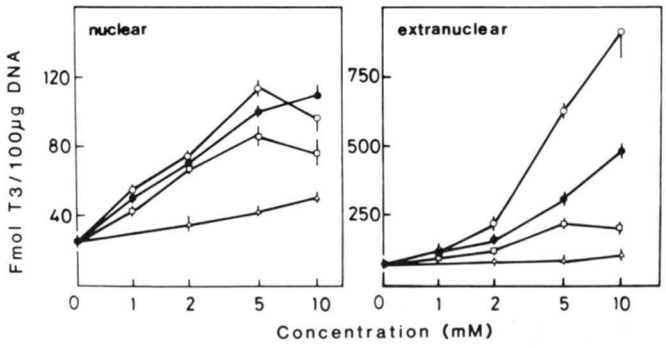

FIG. 7. **Influence of short-chain fatty acids on nuclear receptor and extranuclear T3 levels.** C6 cells were incubated with the concentration of the fatty acids indicated for 48 h. The cultures then received a 0.8 nM concentration of [^{125}I]T3 for 90 min for the determination of nuclear receptor (*left panel*) and extranuclear T3 (*right panel*). The short-chain fatty acids used were the following: butyrate (○); valerate (●); propionate (□); acetate (△).

acids of chain lengths varying between 2 and 5 carbon atoms is shown in Fig. 7. The level of receptor and extranuclear hormone was studied after a 48-h incubation with concentrations ranging between 1 and 10 mM. All compounds had effects qualitatively similar to butyrate, increasing nuclear receptor in a dose-dependent manner. Butyrate induced the receptor maximally at a 5 mM concentration, and valerate was almost as effective reaching the same maximal effect at 10 mM. Propionate and acetate were less potent than butyrate. The same rank order was more clearly observed in the elevation of extranuclear T3 levels, but the hormone content still greatly increased between 5 and 10 mM butyrate.

Fig. 8 compares the effect of a 48-h incubation with butyrate (2–10 mM) with that of similar concentrations of ketone bodies. The insertion of polar groups into the molecule abolishes the effect of the fatty acid, since β-hydroxybutyrate was ineffective. Acetoacetate did not alter either the nuclear receptor or extranuclear [^{125}I]T3 level. In agreement with the data shown in Fig. 7, the effect of butyrate on the receptor was maximal at 5 mM, whereas extranuclear T3 was higher at 10 than at 5 mM.

Influence of Butyrate and Other Short-chain Fatty Acids on Histone Acetylation—The potency of the different fatty acids in increasing nuclear and extranuclear binding parallels the relative inhibitory effects of these compounds on the nuclear deacetylases (16), which suggests that the effects observed by us might be explained by a modification of chromatin structure secondary to acetylation. However, as can be observed in

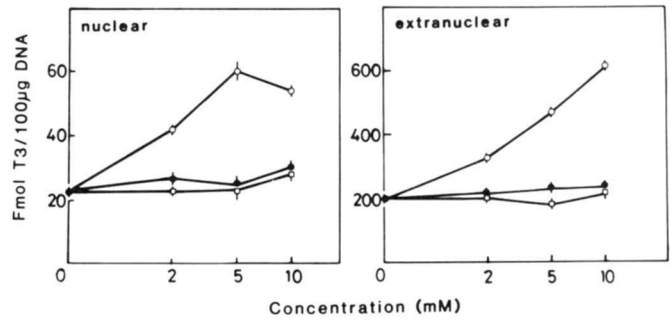

FIG. 8. **Comparison of the effect of butyrate and ketone bodies on nuclear and extranuclear T3 levels.** C6 cells were incubated for 48 h with butyrate (○), β-hydroxibutyrate (●), or acetoacetate (□) at the concentrations indicated. Nuclear (*left panel*) and extranuclear (*right panel*) [^{125}I]T3 binding was determined as described under "Experimental Procedures."

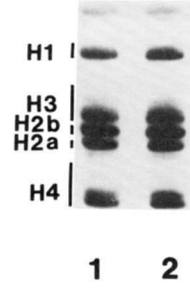

FIG. 9. **Electrophoretic pattern of histones.** C6 cells were incubated for 48 h with (*1*) or without (*2*) 2 mM butyrate, the histones were then extracted and electrophoresed in acid-urea gels as described under "Experimental Procedures."

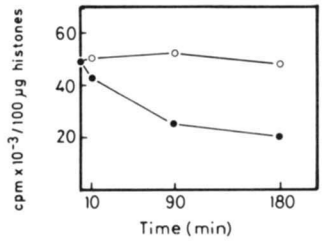

FIG. 10. **Turnover of [^{3}H]acetate associated with the histones.** C6 cells were pulse-labeled for 60 min with 1 mCi/ml [^{3}H] acetate and chased for 10, 90, or 180 min in the absence (●) or presence (○) of 5 mM butyrate. The nuclei were isolated and the histones extracted as described under "Experimental Procedures." An aliquot of the histone fraction was used for determination of radioactivity and the counts per minute were normalized for the amount of histone determined in other aliquot.

Fig. 9, the effect of butyrate on histone acetylation is not easily identified in C6 cells when merely studied by electrophoresis in acid-urea gels, since this compound did not produce the appearance of multiacetylated species of histones H4 and H3. The histones represented in the figure correspond to cells incubated with 2 mM butyrate for 48 h, but a similar finding was observed either with different time periods of incubation with butyrate or with the different fatty acids.

We then studied histone deacetylation in C6 cells by pulse and chase experiments with [^{3}H]acetate. Fig. 10 shows the kinetics of turnover of [^{3}H]acetate incorporated into histones in cells chased in the absence or the presence of 5 mM butyrate. 12% of the radiolabeled acetate was removed after 10 min and about 50% within the first 90 min of the chase in untreated cells. Butyrate drastically reduced the turnover of [^{3}H]acetate, since even after a 180 min chase the whole radiolabel was still incorporated into the histone fraction.

The pattern of Amido Black staining of histones from cells pulsed with [^{3}H]acetate and chased for 90 min in the presence or absence of 2 mM butyrate is shown in Fig. 11 *A* and the corresponding fluorogram in Fig. 11 *B*. About 60–70% of the label was associated to H3 and 20–25% to H4 in all groups. It can be observed in Fig. 11 *B* (*lane 1*) that after the pulse the label is incorporated primarily into the mono- and diacetyl forms of histone H4, although multiacetylated forms are also detected. After the chase with butyrate, a shift to higher acetylated forms was apparent (Fig. 11 *B*, *lane 3*). It should be pointed out that the unacetylated and monoacetylated forms of H4 are the predominant species in the stained gel (Fig. 11 *A*) and that the multiacetylated forms are not detected after the 90 min chase with butyrate, in agreement with the results shown in Fig. 9.

The incorporation of radioactivity after the pulse with [^{3}H] acetate was similar in control cells and in cells previously incubated with 2 mM butyrate for 48 h (47,502 and 53,672 cpm/100 μg of histones, respectively), indicating that whereas the deacetylation is strongly inhibited, the acetylation reaction is not apparently affected by butyrate.

To analyze whether or not the effect of butyrate on T3 binding is related to the degree of histone deacetylation, we examined the effect of butyrate concentrations on [^{3}H]acetate turnover, studied after 1 h pulse and 180 min chase. Fig. 12 illustrates that increasing butyrate concentrations result in a progressive inhibition of histone deacetylation. A half-maximal inhibition was obtained at approximately 1.1 mM butyrate and the inhibition was maximal with 5 mM butyrate, which is in good agreement with the effects on the receptor.

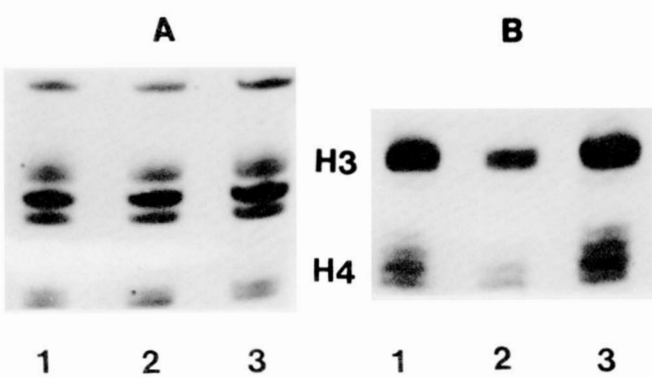

FIG. 11. **Acetylation pattern of the individual histones.** C6 cells were pulsed with [^{3}H]acetate and chased for 90 min in the presence or absence of 2 mM butyrate. Incorporation of acetate was 49,164 cpm/100 μg histones after the pulse, 25,589 cpm/100 μg histones in the chase without butyrate, and 59,718 cpm/100 μg histones after the chase with butyrate. The histones were electrophoresed in acid-urea slab gels, stained with Amido Black and subjected to fluorography. *A*, stained gel. *B*, fluorogram of gel in *A*. *Lane 1*, pulse. *Lane 2*, control chase. *Lane 3*, butyrate chase.

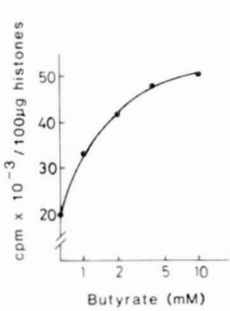

FIG. 12. **Effect of butyrate concentrations on histone acetylation.** C6 cells were pulsed 60 min with [^{3}H]acetate and chased for 180 min in medium containing the butyrate concentrations indicated. Histones were isolated and assayed for protein and radioactivity. The amount of radioactivity incorporated after the pulse was 47,502 cpm/100 μg histones.

The idea that the effect of butyrate on [^{125}I]T3 binding is mediated by a mechanism which involves an inhibition of deacetylation is further supported by a study in which we examined the influence of butyrate and other analogs on both [^{3}H]acetate turnover and receptor levels (Fig. 12). The level of the thyroid hormone nuclear receptor was determined in control cells and after incubation for 48 h with a 5 mM concentration of butyrate, valerate, propionate, isobutyrate, acetate, and α-OH-butyrate. The effect on histone deacetylation was assessed in parallel cultures which were pulsed with [^{3}H]acetate and chased for 180 min with the same concentrations of these compounds. The rank order as well as the potency of the different compounds in both phenomena was very similar and there was a good correlation ($r = 0.93$, $p < 0.01$) between receptor levels and [^{3}H]acetate incorporation into histones (Fig. 13 *A*). The effect of the different compounds on the inhibition of acetate turnover on each individual histone was also examined after gel electrophoresis. When the specific activity of individual H3 or H4 was plotted against receptor levels (Fig. 13, *B* and *C*) there was also a very good correlation ($r = 0.96$, $p < 0.001$, and $r = 0.97$, $p < 0.01$, respectively) in agreement with the data shown in Fig. 13 *A* in which the incorporation into the total histone fraction is represented.

DISCUSSION

After the entry of thyroid hormones into the cell, their action appears to be mediated through binding to the nuclear receptor, a chromatin-associated protein (1). Changes in hormone uptake, in the amount of T3 reaching the nucleus, and/or in receptor levels may be critical factors in modulating the sensitivity of the cell to the hormone.

In this study we have examined the effect of butyrate, and other short-chain fatty acids, on T3 binding to the nuclear and extranuclear compartment of C6 cells, a rat glioma cell line. Our data clearly demonstrate that, in contrast with the effect found in GH1 cells (21), butyrate 1 mM and higher causes a time- and dose-dependent increase of both nuclear and extranuclear T3 levels in C6 cells. That the effect of butyrate on nuclear binding is not simply a reflection of the higher total cellular T3 content is strongly suggested by the Scatchard analysis of T3 nuclear binding in intact cells and is further demonstrated by the finding that an increase in maximal binding capacity is also found when binding was studied in isolated nuclei, in the absence of interference from extranuclear T3. Therefore, we conclude that butyrate affects extranuclear T3 levels in C6 cells; and that at the same time, but possibly independently, there is an increase in nuclear receptor without a change in receptor affinity.

The rise in receptor levels could be a consequence of an increased synthesis, a decreased degradation, or a combination of both. We have not directly measured the rate of receptor synthesis, but our data suggest that butyrate acts primarily by decreasing receptor degradation. The mechanism(s) underlying the extranuclear T3 increase have not yet been defined, but it must represent a rise in hormone uptake and/or in the binding of T3 to the cytoplasmic compartment. Whether or not butyrate influences membrane or cytosolic T3-binding proteins awaits further investigation, but it is likely that a reduced turnover of extranuclear-binding proteins, as suggested for the nuclear receptor, might be also responsible for the increased extranuclear levels.

Since butyrate is known to be a reasonably efficient synchronizer of cells into G1 phase (5, 6), the increase of receptor

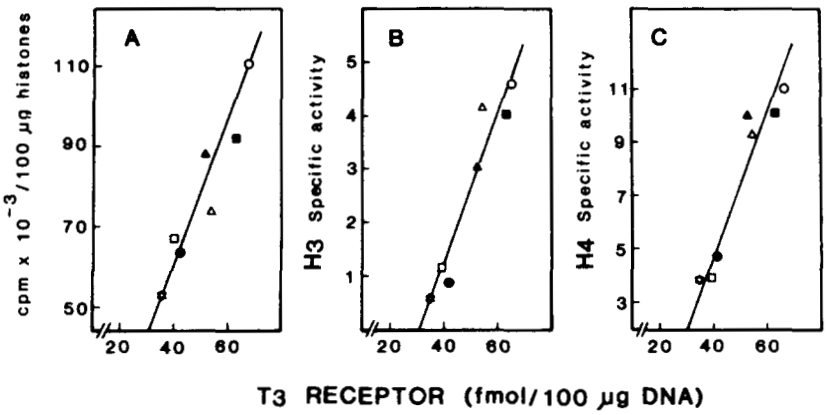

FIG. 13. **Relationship of nuclear receptor levels to histone acetylation.** T3 receptor levels were determined in control cells (●) and after 48 h incubation with a 5 mM concentration of butyrate (○), valerate (■), propionate (▲), isobutyrate (△) acetate (□) and α-hydroxibutyrate (●). Histone acetylation was assessed by pulse-labeling parallel sets of cultures with [^{3}H]acetate and chasing for 180 min in the presence of the same concentration of the different compounds. Histones were isolated and assayed for protein and radioactivity. Nuclear receptor levels obtained with each analog are plotted against the [^{3}H]acetate remaining in each group after the chase period (*panel A*). The histones were then extracted and electrophoresed in acid-urea slab gels. The gels were dried and the amount of histone applied assessed by densitometric scanning of the Amido Black-stained gel. The individual histone bands were cut out and counted, and the area of the corresponding stained peak calculated. The ratio area/[^{3}H]acetate for histones H3 (*panel B*) and H4 (*panel C*) was plotted against receptor levels.

or extranuclear T3 levels may simply be a cell cycle-dependent phenomenon. It has been shown in pituitary GC cells that the levels of T3 receptor vary among the different stages of the cell cycle (38). However, the changes were very small (only 30–50%) compared to those observed by us, making this mechanism very unlikely for our observations in C6 cells. In addition, there are concentrations of fatty acids that increase receptor levels without changing total DNA/culture. This is especially striking with isobutyrate which can increase receptor levels by 2–3-fold without altering either cell proliferation or extranuclear T3 levels.[2] Therefore, an inhibition of cell replication does not seem to be the cause of the effect on the receptor, although we do not discard an influence of the inhibition of DNA synthesis on extranuclear T3.

The increase of receptor levels caused by butyrate in C6 cells is quantitatively very important. This cell line has a relatively low concentration of T3 receptors (20–30 fmol/100 μg DNA). However, after butyrate treatment of C6 cells receptor concentration increases severalfold reaching up to 90–120 fmol/100 μg DNA. These levels are even higher than those normally found in rat liver (50–100 fmol/100 μg DNA) and almost reach the concentrations of receptor present in pituitary GH cells (100–200 fmol/100 μg DNA), which possess the highest level of thyroid hormone receptors reported so far.

It is normally assumed that, within a given cell type, there is some type of relationship between the levels of hormone-receptor complexes and the degree of action exerted by the hormone. Although we have not measured the effect of butyrate on a thyroid hormone-mediated action in C6 cells, we would thus expect an increased biological action of T3 after treatment with butyrate. Moreover, if as proposed by Oppenheimer and co-workers (39, 40) there is a concentrating mechanism governing the transport of T3 from medium to cytoplasm and from cytoplasm to nucleus, the increase in extranuclear hormone produced by butyrate might lead to an increased receptor occupancy and enlarge the sensitivity of C6 glial cells to thyroid hormones. An augmentation of hormonal effect would not, however, occur if butyrate increases binding proteins without altering free extranuclear T3. We do not know yet whether the regulation of receptors and extranuclear levels also occurs in nontransformed glial cells, but if this is the case it would not be surprising that short-chain fatty acids might play a basic role in the development or maintenance of receptor levels and in the responsiveness of glial cells to thyroid hormone during and/or after brain development.

The effect of butyrate and other analogs on total cellular T3 levels followed a pattern similar to that of the effect on nuclear binding but with several differences: (i) whereas the effect of butyrate on the receptor was maximal at 5 mM, extranuclear T3 still increased between 5 and 10 mM (Figs. 7 and 8). (ii) It takes a longer time to observe the increase in extranuclear T3, since after short incubation times with butyrate (for example, 6 h) a small but detectable increase of receptor is observed but no effect on extranuclear T3 is found. (iii) The effect on the receptor is rapidly reversed after butyrate removal, whereas extranuclear T3 levels are very slowly restored to normal. (iv) Although the rank order of the different fatty acids on nuclear and cellular hormone levels is qualitatively similar, there are important quantitative differences on the magnitude of both responses with different concentrations of the fatty acids (Fig. 7). This suggests that different mechanisms could be responsible for the butyrate regulation of nuclear and extranuclear T3 levels, and we cannot even exclude that both effects could be mediated by actions of butyrate on different cell components.

Butyrate is known to profoundly affect chromatin structure and function (8), therefore the regulation of receptor might be secondary to an action of butyrate on regions of the chromatin to which the receptor is bound. We have studied in C6 cells the best known action of butyrate, namely the inhibition of histone deacetylation (14–22), and have tried to correlate this biochemical effect with the regulation of receptor levels. In C6 cells butyrate and other fatty acids did not appreciably increase the level of multiacetylated forms of nucleosomal core histones as studied by gel electrophoresis. This was totally unexpected since as much as 80% of H4 can be modified in several mammalian cell types after exposure to butyrate (4, 14, 18, 21, 41). Since in C6 cells butyrate inhibited [^{3}H]acetate turnover, the apparent lack of effect on

[2] J. Ortiz-Caro and A. Aranda, unpublished observations.

histone acetylation probably reflects that in C6 cells a large percentage of core histone complement remains unmodified even after exposure to the fatty acids. If so, a shift of protein into high acetylation levels would not be identified since the amount of protein would be too small to be detected by gel staining. Alternatively, the small percentage of modified histones in the presence of butyrate could be explained by a reduction of histone acetylation after exposure to this compound. Treatment of hepatoma cells with butyrate results in significantly reduced amounts of histone acetyltransferase (41) and in a reduced level of overall incorporation of [^{3}H] acetate (16), but incubation with butyrate for 48 h did not change [^{3}H]acetate incorporation in C6 cells in agreement with findings in HeLa cells (4). Therefore, a predominant effect of butyrate on inhibition of acetylation over deacetylation seems unlikely.

In C6 cells the deacetylation rate (Fig. 9) was also very slow when compared to other cell types (16, 18, 19, 41). This exceedingly slow deacetylation agrees, however, with the data obtained by Kumar *et al.* (13) which found that histones from C6 cells pulsed with [^{3}H]acetate for 15 min still retain 10% of the label after an 8 h chase and 5% after 24 h.

Our data suggest a close relationship between the inhibition of histone deacetylation and the modulation of receptor levels in C6 cells, since we found a very good correlation between the increase of receptor levels caused by the different fatty acids and the inhibition of [^{3}H]acetate release from the total histone fraction, or from individual H3 and H4. In addition, there was a striking similarity in the dose-response of butyrate for increase of receptor levels and inhibition of histone deacetylation. Therefore, our studies strongly suggest that the butyrate-mediated increase in thyroid hormone receptor levels in C6 cells could be explained by a modification of chromatin structure secondary to acetylation. However, we cannot exclude the possibility that acetylation of other nonhistone chromatin proteins, or even other type of modification of nuclear proteins (22), could be responsible for the modulation of receptor levels.

The question is then: why butyrate through the same hypothetical mechanism produces an opposite effect on thyroid hormone receptor in C6 and GH1 cells? There are several possible explanations. We have previously described in GH1 cells the existence of at least two subunits of receptor (26) which are regulated differently. Butyrate decreases receptor number in GH1 cells by increasing the degradation of the abundant (approximately 90% of the total) 47,000 M_r form, but in addition, at low concentrations (0.5 mM or less), it produces a small increase of receptor levels which appears to be due to an increase of the minor 57,000 M_r form (27). A higher proportion of the 57,000 M_r form in C6 cells might certainly contribute to explain the specific control that butyrate exerts on the T3 receptors in both types of cells. Experiments using a photoaffinity label probe (26) will hopefully clarify the role of both forms on the regulation of receptor number by butyrate in C6 cells.

Additionally, the different behavior may also reflect a different sensitivity to the fatty acid. C6 cells appear to be less sensitive than GH1 cells, 0.05–0.1 mM butyrate causes the maximal increase of receptor levels in GH1 cells (28), whereas in C6 cells a maximal effect on receptor increase is found at 5 mM, a 50–100-fold higher concentration. Butyrate reduces receptor levels in GH1 cells at 1 mM and above, and we might need concentrations of at least 50–100 mM to reduce the receptor in C6 cells. These differences between the glial and pituitary cell lines may be related to the quantitative differences found in histone acetylation. In GH1 cells butyrate produces a massive hyperacetylation of core histones which is reflected by a quantitatively important increase of multiacetylated forms of histones H4 and H3 when analyzed by electrophoresis in acid-urea gels (21), whereas as suggested above, histones in C6 cells appear to be less susceptible to acetylation.

In conclusion, our data are compatible with a model in which the modification of a relatively small proportion of nucleosomes would produce a change in chromatin organization which leads to a decrease of receptor degradation and as a consequence to an increase of steady-state levels of receptor. This would occur in C6 cells after exposure to butyrate and when GH1 cells are incubated with low concentrations of butyrate. By contrast, acetylation of a high proportion of nucleosomal proteins would result in a conformational change in the chromatin which would shorten the half-life of the receptor and reduce receptor levels. This would be the case in GH1 cells incubated with high concentrations of butyrate.

Acknowledgment—We thank Dr. G. Morreale de Escobar for the critical revision of this manuscript and Aida Villa for her excellent technical assistance.

REFERENCES

1. Appriletti, J. W., David-Inouye, Y., Baxter, J. D., and Eberhardt, N. L. (1983), in *Molecular Basis of Thyroid Hormone Action* (Oppenheimer, J. H., and Samuels, H. H., eds) pp. 67–96, Academic Press, New York
2. Kruh, J. (1982) *Mol. Cell. Biochem.* **42,** 65–82
3. Wright, J. A. (1973) *Exp. Cell Res.* **78,** 456–460
4. Hagopian, H. K., Riggs, M. G., Swartz, L. A., and Ingram, V. M. (1977) *Cell* **12,** 855–860
5. Van Wijk, R., Tichonicky, L., and Kruh, J., (1981) *In Vitro (Rockville)* **17,** 859–862
6. Xue, S., and Rao, P. N. (1981) *J. Cell. Sci.* **51,** 163–171
7. Reeves, R., and Cserjesi, P. (1979) *J. Biol. Chem.* **254,** 4283–4290
8. McKnight, G. S., Hager, L., and Palmiter, R. D (1980) *Cell* **22,** 469–477
9. Covault, J., Perry, M., and Chalkley, R. (1982) *J. Biol. Chem.* **257,** 13433–13440
10. Plesko, M. M., Hargrove, J. L., Granner, D. K., and Chalkley, R. (1983) *J. Biol. Chem.* **258,** 13738–13744
11. Leibovitch, M. P., Leibovitch, S. A., Raymondjean, M., and Kruh, J. (1984) *Biochem. Biophys. Res. Commun.* **125,** 1129–1136
12. Ginder, G. D., Whitters, M. J., and Pohlman J. K. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 3954–3958
13. Kumar, S., Weingarten, D. P., Callahan, J. W., Sachar, K., and de Vellis, J. (1984) *J. Neurochem.* **43,** 1455–1463
14. Riggs, M. G., Whittaker, R. G., Neumann, J. R., and Ingram, V. M. (1977) *Nature* **268,** 462–464
15. Boffa, L. C., Vidali, G., Mann, R. S., and Allfrey, V. G. (1978) *J. Biol. Chem.* **253,** 3364–3366
16. Sealy, L., and Chalkley, R. (1978) *Cell* **14,** 115–121
17. Sterner, R., Vidali, G., and Allfrey, V. G. (1981) *J. Biol. Chem.* **256,** 8892–8895
18. Cousens, L. S., Gallwitz, D., and Alberts, B. M. (1979) *J. Biol. Chem.* **254,** 1716–1723
19. Covault, J., and Chalkley, R. (1980) *J. Biol. Chem.* **255,** 9110–9116
20. Candido, E. P., Reeves, R., and Davie, J. R. (1978) *Cell* **14,** 105–113
21. Samuels, H. H., Stanley, F., Casanova, J., and Shao, T. C. (1980) *J. Biol. Chem.* **255,** 2499–2508
22. Boffa, L. C., Gruss, R. J., and Allfrey, V. G. (1981) *J. Biol. Chem.* **256,** 9612–9621
23. Borefreund, E., Schmid, E., Bendich, A., and Franke, W. W. (1980) *Exp. Cell. Res.* **127,** 215–235
24. Fishman, P. H., Simmons, J. L., Brady, R. O., and Freese, E. (1974) *Biochem. Biophys. Res. Commun.* **59,** 292–299
25. Kassis, S., Henneberry, R. C., and Fishman, P. H. (1984) *J. Biol. Chem.* **259,** 4910–4916
26. Pascual, A., Casanova, J., and Samuels, H. H. (1982) *J. Biol. Chem.* **257,** 9640–9647

27. Casanova, J., Horowitz, Z. D., Copp, R. P., McIntyre, W. R., Pascual, A., and Samuels, H. H. (1984) *J. Biol. Chem.* **259,** 12084–12091
28. Stanley, F., and Samuels, H. H. (1984) *J. Biol. Chem.* **259,** 9768–9775
29. Ortiz-Caro, J., Yusta, B., Montiel, F., Villa, A., Aranda, A., and Pascual, A. (1986) *Endocrinology*, in press
30. Weingarten, D. P., Kumar, S., Bressler, J., and de Vellis, J. (1984) in *Advances in Neurochemistry*, (Norton, W., ed). pp. 299–338, Plenum Press, New York
31. Samuels, H. H., Stanley, F., and Casanova, J. (1979) *Endocrinology* **105,** 80–85
32. Samuels, H. H., and Tsai, J. R. (1973) *Proc. Natl. Acad. Sci. U. S. A.* **70,** 3488–3492
33. Samuels, H. H., Stanley, F., and Shapiro, L. E. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73,** 3877–3881
34. Panyim, S., and Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130,** 337–345
35. Chamberlain, J. P. (1979) *Anal. Biochem.* **98,** 132–135
36. Burton, K. (1956) *Biochemistry* **62,** 315–323
37. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
38. DeFesi, C. R., Fels, E. C., and Surks, M. I. (1982) *Endocrinology* **111,** 1156–1165
39. Oppenheimer, J. H., and Schwartz, H. L. (1985) *J. Clin. Invest.* **75,** 147–154
40. Freake, H. C., Mooradian, A. D., Schwartz, H. L., and Oppenheimer, J. H. (1986) *Mol. Cell. Endocr.* **44,** 25–35
41. Covault, J., Sealy, L., Scnell, R., Shires, A., and Chalkley, R. (1982) *J. Biol. Chem.* **257,** 5809–5815