Sodium Butyrate in Combination with Insulin or Dexamethasone Can Terminally Differentiate Actively Proliferating Swiss 3T3 Cells into Adipocytes*

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Sodium butyrate arrests the growth of actively proliferating Swiss 3T3 cells. A previous report from our laboratory describes the pattern of expression of a representative group of growth-associated genes following treatment of Swiss 3T3 cells with sodium butyrate. The results of this study suggest that sodium butyrate-induced growth arrest involves events which lead to adipocyte differentiation (Toscani, A., Soprano, D. R., and Soprano, K. J. (1988) Oncogene Res. 3, 233–238). However, while sodium butyrate by itself could apparently initiate adipogenesis, it alone was not sufficient to maintain this differentiation state. We now wish to further characterize the role of sodium butyrate in adipocyte differentiation. Subconfluent cultures of Swiss 3T3 cells were treated with sodium butyrate in combination with other agents known to induce Swiss 3T3 cell adipogenesis (e.g. 1-methyl-3-isobutylxanthine, insulin, and dexamethasone) and then analyzed at various times thereafter for: (a) the presence of high concentrations of intracellular lipid as detected by microscopic examination of treated cells following staining with lipid-specific dyes and (b) the expression of four genes known to be modulated during the differentiation of preadipocytes into mature adipocytes (actin, adipin, lipoprotein lipase, and adipocyte P2). Our results show that sodium butyrate in combination with either insulin or dexamethasone can fully differentiate Swiss 3T3 cells into adipocytes, at least as determined by accumulation of high levels of intracellular lipid. Moreover, the sodium butyrate-mediated process of differentiation can occur in subconfluent, actively proliferating cells. Thus, these experiments describe a new, previously unidentified activity of sodium butyrate and also suggest that this model system may be a useful one to study the relationship between growth arrest and differentiation.

Sodium butyrate is a pharmacological agent known to have various antiproliferative effects on cultured animal cells (1–5). For example, millimolar concentrations of sodium butyrate added to cultures of actively proliferating cells lead to: (a) an inhibition of DNA synthesis by arresting these cells in the G1 phase of the cell cycle (2, 4); (b) a modulation of the expression of a variety of genes (3, 6–9); and (c) an induction of terminal differentiation (1, 3, 10–12). However, while it is well established that sodium butyrate treatment results in growth arrest of cells in culture, the mechanism(s) by which sodium butyrate exerts these various effects is generally not known.

Swiss mouse 3T3 cells have been shown to be capable of spontaneously differentiating into adipocytes after they have become density/growth-arrested. The process takes 2–4 weeks and the percentage of cells converting to adipocytes varies from culture to culture. Cell lines which convert to adipocytes at a high frequency have been clonally isolated from the original Swiss 3T3 cell cultures (i.e. 3T3-L1 and 3T3-F442A) (13, 14). In addition, a variety of agents have been identified which can accelerate the differentiation process of 3T3-L1 and 3T3-F442A cells. These include 1-methyl-3-isobutylxanthine (MIX) (15), insulin (16), dexamethasone (17), prostaglandin F2α (18), and high serum concentration (20–30%) (19). One of the most efficient means to induce adipogenesis in either 3T3-L1 or 3T3-F442A cells was treatment of confluent cultures of 3T3-L1 cells with a combination of MIX, insulin, and dexamethasone (17, 20, 21). This particular strategy resulted in conversion of 85–90% of the cells to adipocytes within 7 days after reaching the confluent state (17).

We have previously examined sodium butyrate-mediated growth arrest in Swiss 3T3 mouse fibroblast cells at the molecular level in order to better understand the nature of this particular type of growth arrest. Analysis of the expression of several growth-associated genes (thymidine kinase, c-myc, p53, and c-fos) and one adipocyte-specific gene (adipocyte P2 (aP2)) at various times after treatment of Swiss 3T3 cells with sodium butyrate provided evidence that sodium butyrate arrest of Swiss 3T3 cell growth might involve one or more of the events which accompany the conversion of these cells into adipocytes. However, these experiments also suggest that sodium butyrate by itself was not sufficient to maintain this differentiated state. We now wish to extend these studies in order to identify the precise role (if any) that sodium butyrate plays in Swiss 3T3 cell adipogenesis. More specifically, we chose to determine whether sodium butyrate could substitute for one or more of the agents previously shown to induce adipocyte differentiation in Swiss 3T3 cells.

In these studies, we used two criteria for adipocyte differentiation: (a) the accumulation of high concentrations of intracellular lipid as detected microscopically using lipid-specific dyes (Oil Red O and Nile Red) and (b) expression of genes known to be modulated during adipocyte differentiation (actin, adipin, lipoprotein lipase, and aP2). Our results show that sodium butyrate in combination with either insulin or...
dexamethasone can mediate the complete, terminal differentiation of Swiss 3T3 cells into adipocytes. Moreover, in contrast to more classical methods, the sodium butyrate-mediated process can occur in subconfluent cultures. These experiments therefore describe a new, previously unidentified activity of sodium butyrate. In addition, they describe a model system which can be used to help elucidate the relationship between growth arrest and differentiation.

**MATERIALS AND METHODS**

**Cell Culture**—Swiss 3T3 cells were obtained from Dr. Renato Baserga (Department of Pathology, Temple University). Stock cultures of Swiss 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics (Hazelton, Denver, PA) at 37 °C in a humidified atmosphere containing 10% CO2.

**Cell Culture Treatments**—Cells were plated in 100-mm tissue culture dishes (Corning Glass Works, Corning, NY) in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at a density of 1 × 10^6 cells/cm². Twenty-four h after plating, the cell cultures were treated with various agents either in the absence or presence of sodium butyrate (Sigma) at a final concentration of 5 mM. The treatments were as follows: (a) no treatment, (b) only sodium butyrate, (c) MIX (Sigma) at a final concentration of 115 µg/ml, (d) MIX + sodium butyrate, (e) insulin (Sigma) at a final concentration of 10 µg/ml, (f) insulin + sodium butyrate (g) dexamethasone (Sigma) at a final concentration of 390 ng/ml, and (h) dexamethasone + sodium butyrate.

In another experiment, the cells were allowed to reach confluence (5 days after plating). These confluent cells were then treated as described by Bernlohr et al. (21) in the following manner: first, the cells were treated for 48 h with MIX (115 µg/ml) + insulin (10 µg/ml) + dexamethasone (390 ng/ml). After this 48-h period, the medium was replaced with fresh medium containing insulin (10 µg/ml).

**Lipid Staining of Cells**—Swiss 3T3 cells were grown and treated in duplicate cultures as described above in 60-mm tissue culture dishes. After the treatments, the cell cultures were fixed in isotonic phosphate buffer and one set of cultures was stained with Nile Red O as described by Kuri-Harcuch and Green (22) and the other set of cultures was stained with Nile Red as described by Greenspan et al. (23).

**Cellular RNA Isolation**—At the end of the various treatments, cells were harvested by scraping. Total cytoplasmic RNA was isolated by the method of Chirgwin et al. (24) and was quantitated by determination of the absorbance at 260 nm. The RNA solution was then denatured and separated on polyacrylamide-urea gels. Gels were dried and exposed to Kodak SB-5 film with intensifying screens at -80 °C. Bands were scanned from appropriate exposures of the autoradiographs with a Hoefer model 1650 scanning densitometer and quantitated by determination of the area under each peak with a planimeter.

**Northern Blot Analysis**—In some experiments, total cytoplasmic RNA (35 µg) was analyzed on 1% agarose-formaldehyde gels as described by Lehrach et al. (32). The conditions for transfer of RNA to nitrocellulose, prehybridization, hybridization, and subsequent washing were as described previously by Soprano et al. (30).

**Autoradiography**—DNA synthesis was assayed in individual cells following treatment with various adipogenic agents by autoradiography. Cells were grown on glass coverslips and labeled at the indicated times by addition to the medium of [3H]thymidine (25 Ci/mmol; Amersham Corp.) at a final concentration of 0.1 µCi/ml. The cells were then fixed in absolute methanol and processed as described previously (25, 26).

**RESULTS**

We previously showed that in actively proliferating Swiss 3T3 cells, the steady-state mRNA levels of c-fos and aP2 were undetectable but were induced upon treatment of these proliferating cells with sodium butyrate (33). Both genes, c-fos and aP2, were induced early (1–3 days) after treatment with sodium butyrate but their levels were not maintained when treatment was extended for longer periods of time (7 days). These results suggest that while sodium butyrate was able to initiate events involved in the differentiation of Swiss 3T3 cells into mature adipocytes, it could not maintain them and thus did not induce terminal adipogenesis.

Since treatment with a combination of MIX, insulin, and dexamethasone can completely convert density/growth-arrested Swiss 3T3 cell lines such as 3T3-L1 and 3T3-F442A to terminal adipocytes, and since sodium butyrate rapidly arrests subconfluent, actively proliferating Swiss 3T3 cells, we wondered whether terminal Swiss 3T3 adipogenesis could be induced in subconfluent, actively proliferating cells by treatment with a combination of sodium butyrate and one or more of these agents.

**Analysis of Intracellular Lipid**—We chose to initially monitor adipogenesis by determination of the accumulation of high concentrations of intracellular lipid, the major phenotypic and morphological characteristic of adipocytes. We analyzed the presence of lipids in the cells at various days following treatment microscopically after staining with the lipid-specific dyes: Oil Red O (22, 35) and Nile Red (23, 36). Oil Red O is a dye that has been traditionally used in the studies of adipocyte differentiation. Morphological analysis of lipoid accumulation has been shown to correlate with the differentiation state of the cell (13, 14, 22, 34). Nile Red is a fluorescent dye which has also been shown to be highly selective and specific for neutral lipids (23, 36).

Representative micrographs of Swiss 3T3 cells stained with Nile Red 7 days following various treatments, the time of maximum lipid accumulation (see below and Fig. 3), are shown in Fig. 1. We have previously shown (35) that non-treated, subconfluent cultures of Swiss 3T3 cells contain virtual no cells which stain intensely with Nile Red. However, Fig. 1A shows that a limited number of cells in non-treated, confluent (growth-arrested) cultures of Swiss 3T3 cells exhibit intense staining, indicating that they contain high levels of intracellular lipid. The positive control culture (density arrested cells treated with MIX + insulin + dexamethasone), which contains numerous lipid-positive cells, is shown in Fig. 1B. Subconfluent cultures treated with sodium...
butyrate alone, insulin alone, or dexamethasone alone were found to contain more lipid-positive cells than nontreated subconfluent cultures but not as many as was observed in the positive control cultures (data not shown; however, see Toscani et al. (33) and see below for quantitation). However, a considerably higher number of lipid-positive cells were seen in cultures treated with both sodium butyrate and dexamethasone (Fig. 1C) or sodium butyrate and insulin (Fig. 1D). It should be noted that both the overall representation of these cells in the treated population and the intensity of staining of individual cells was comparable to those obtained by the standard method of inducing adipocyte differentiation (treatment of density-arrested cells with MIX, insulin, and dexamethasone, see Fig. 1B).

Our results with Nile Red were confirmed by Oil Red O staining and were quantitated by determination of the percentage of cells containing numerous, intensely stained lipid droplets (“lipid-positive”). These data are summarized in Table I. It is known that all cultures of Swiss 3T3 cells contain numerous, intensely stained lipid droplets (“lipid-positive”). These data are summarized in Table I. It is known that all cultures of Swiss 3T3 cells contain numerous, intensely stained lipid droplets (“lipid-positive”). These data are summarized in Table I. It is known that all cultures of Swiss 3T3 cells contain numerous, intensely stained lipid droplets (“lipid-positive”).
butyrate alone; W, sodium butyrate + dexamethasone; +, sodium butyrate alone. However, the maximum response was obtained especially in combination with insulin. Even higher numbers increase in the number of Oil Red O-positive staining cells, at least as determined at 7 days following treatment.

**Time Course of Lipid Accumulation during Sodium Butyrate-mediated Adipocyte Differentiation**—We next wished to compare the time of appearance of high concentrations of intracellular lipid in response to treatment of actively proliferating Swiss 3T3 cells with either 5 mM sodium butyrate alone or 5 mM sodium butyrate in combination with insulin or dexamethasone. Fig. 3 shows that the highest percentage of Oil Red O-positive cells was observed at 7 days regardless of treatment. However, the cells treated with sodium butyrate and insulin continued to maintain high levels in intracellular lipid through 17 days, the last time point analyzed. In contrast, the cells treated either with sodium butyrate alone or sodium butyrate in combination with dexamethasone appeared to lose their Oil Red O-staining material between day 10 and day 12. These data suggest that terminal adipocyte differentiation, at least as defined by maintenance of high concentrations of intracellular lipid was induced only by treatment with sodium butyrate and insulin.

**Sodium Butyrate-mediated Adipocyte Differentiation Is Terminal**—We next wished to determine if this sodium butyrate-mediated differentiation event was terminal. The criteria used to determine this was continued presence of lipid and failure to reenter the cell cycle upon removal of the differentiation agents. Swiss 3T3 cells were treated with either sodium butyrate alone, sodium butyrate plus dexamethasone, or sodium butyrate plus insulin. After 7 days, either or both agents were removed and the cells were incubated for an additional 7 days. The percentage of lipid-positive cells and the percentage of cells synthesizing DNA was then determined.

Table II (column 3) shows that in cells treated with sodium butyrate and insulin, removal of either or both agents did not drastically alter the percentage of lipid-positive cells. We did observe a reduction (~20%) in the number of intensely staining cells following removal of either sodium butyrate alone or both sodium butyrate and dexamethasone. This is consistent with data obtained in the time-course experiment described previously suggesting that terminal differentiation resulted only from treatment with sodium butyrate and insulin and not from treatment with sodium butyrate and dexamethasone. That the lipid-positive cells resulting from treatment with
sodium butyrate and insulin are terminally differentiated and cannot (and do not) reenter a state of active cell proliferation is shown in Table II (columns 4 and 5). Swiss 3T3 cells grown on coverslips were treated for 7 days with either sodium butyrate alone, sodium butyrate plus dexamethasone, or sodium butyrate plus insulin, at which time either or both reagents were removed. At the time of removal, coverslips were incubated in complete medium containing [3H]thymidine. Coverslips were then harvested at either 24 or 72 h and processed for autoradiography. Analysis of the percentage of cells synthesizing DNA shows that DNA synthesis did not occur unless the sodium butyrate was removed. During the first 24 h following removal, approximately 15% of the cells treated either with sodium butyrate alone, with sodium butyrate and insulin, or sodium butyrate and dexamethasone entered DNA synthesis (and thus presumably began to proliferate). By 3 days following removal of the reagents, the percentage of proliferating cells in the cultures previously treated with sodium butyrate alone or sodium butyrate plus dexamethasone increased by nearly 4-fold (61 and 57%, respectively) whereas the percentage of labeled cells in the cultures previously treated with sodium butyrate and insulin was considerably lower (28%). Moreover, cells which were labeled did not exhibit the typical morphology of an adipocyte (i.e. they were relatively small in size, mononucleated and free of vacuoles). These data suggest that Swiss 3T3 cells treated with a combination of sodium butyrate and insulin for 7 days are induced to enter a terminal state of adipocyte differentiation.

Analysis of Adipocyte-specific Gene Expression—A number of genes have been previously shown to be induced and continuously expressed in confluent Swiss 3T3 cells differentiated into adipocytes by the standard treatment. These include adipin, adipocyte P2 (aP2), and lipoprotein lipase (LPL) (21, 37-43). Since actively growing Swiss 3T3 cells treated with either sodium butyrate and insulin or sodium butyrate and dexamethasone exhibited the morphological properties associated with terminal adipocyte differentiation, we next wished to determine if these same cells also exhibited the molecular properties of terminally differentiated adipocytes.

Actively proliferating Swiss 3T3 cells were treated with either MIX, insulin, or dexamethasone in the absence or presence of sodium butyrate as described above. Total cytoplasmic RNA was isolated and the steady-state mRNA levels of the adipocyte-specific genes were analyzed either by Northern blot assay (actin and adipsin) or by RNase protection assay (aP2 and LPL).

Expression of aP2 and Lipoprotein Lipase—Fig. 4 represents a composite of autoradiographs made from polyacrylamide gels used to analyze the steady-state mRNA levels of aP2, LPL, and β2-microglobulin in subconfluent Swiss 3T3 cell cultures following treatment with the various agents. β2-Microglobulin mRNA level served as an internal control for the relative amount of RNA assayed in each of the samples analyzed in these experiments. It can be seen in the bottom panel of Fig. 4 that there was only slight variation in RNA level from sample to sample.

The expression of aP2 has been shown to be induced early during adipocyte differentiation (21, 38-42). Fig. 4A shows that, as expected, aP2 mRNA was not detectable in the nontreated actively growing cultures or in cells treated with MIX or MIX plus sodium butyrate. Likewise, aP2 mRNA expression was induced in subconfluent cells treated with a combination of either sodium butyrate and insulin or sodium butyrate and dexamethasone. However, a low level of aP2 was also surprisingly detected in cultures treated with sodium butyrate alone, insulin alone, and dexamethasone alone despite the fact that none of these treatments produced the morphological changes characteristic of terminal adipocyte differentiation.

Another biochemical marker of adipocyte differentiation is the induction of LPL activity (34, 44-46). However, Fig. 4A (LPL panel), shows that LPL mRNA was detected in nontreated, actively growing Swiss 3T3 cells (lane 1) as well as in all of the cultures treated with the various agents (lanes 2-7). It would appear, therefore, that the expression of both aP2 and LPL may not be restricted only to cells which exhibit the morphological characteristics of terminal adipocyte differentiation (i.e. high concentrations of intracellular lipid).

Expression of Actin and Adipsin—Fig. 5 represents a Northern blot analysis of two additional genes (actin and adipsin) which are known to be modulated in opposite directions during the differentiation of preadipocytes into mature adipocytes. The level of actin mRNA has been previously shown to decrease slightly (less than 2-fold) during the differentia-
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FIG. 4. Steady-state mRNA levels of aP2 and LPL in Swiss 3T3 cells. A, steady-state levels of aP2, LPL, and β₂-microglobulin (β₂-M) mRNA were analyzed by RNase protection assay using total RNA isolated from subconfluent cultures of Swiss 3T3 cells treated with various adipogenic agents as described under “Materials and Methods.” B, quantitative comparison of aP2 mRNA (■) and LPL mRNA (□) levels was obtained by densitometric scanning of appropriate exposures of autoradiographs obtained from the gels in A. The relative integrated density (RID) value obtained was then normalized to β₂-microglobulin mRNA levels. NaB, sodium butyrate; DEX, dexamethasone; INS, insulin.

FIG. 5. Steady-state mRNA levels of actin and adipsin in Swiss 3T3 cells. A, steady-state level of actin and adipsin mRNA was analyzed by Northern blot using total RNA obtained from subconfluent cultures of Swiss 3T3 cells treated with various agents as described under “Materials and Methods.” B, quantitative comparison of actin mRNA was obtained by densitometric scanning of an appropriate exposure of the gel in A. The RID value was normalized to β₂-microglobulin mRNA levels. NaB, sodium butyrate; DEX, dexamethasone; INS, insulin; RID, relative integrated density.

Treatment with insulin, insulin + sodium butyrate, or dexamethasone + sodium butyrate caused only a slight reduction (if any) in actin steady-state mRNA levels.
This same blot was also hybridized to a probe for mouse adipoprotein. The expression of this gene is preferentially induced later in the differentiation of preadipocytes to adipocytes, after the induction aP2 (37, 48). As shown in Fig. 5A, adipoprotein mRNA was only detectable in subconfluent Swiss 3T3 cells that were treated with sodium butyrate + dexamethasone. Surprisingly, we were unable to detect even low levels of adipoprotein mRNA in cultures treated with sodium butyrate and insulin, even when the blots were exposed for extremely long periods of time.

Thus, as was true for aP2 and LPL, the expression of adipoprotein and actin does not appear to be exclusively coupled to the ability of a cell to accumulate high levels of intracellular lipid.

**DISCUSSION**

Four important observations can be made from analysis of the experiments reported here: (a) while sodium butyrate has been previously shown to mediate differentiation in murine embryonal carcinoma cells (49) and a number of cells of neuronal origin including rat sympathetic neurons (50), mouse neuroblastoma cells (51), and rat glioma cells (52), this is the first report to show that sodium butyrate can mediate adipocyte differentiation; (b) sodium butyrate and dexamethasone and sodium butyrate and insulin are among the only commercially available, completely defined agents known which will induce Swiss 3T3 cells to convert to mature adipocytes without prior growth arrest, cell-cell contact or treatment with specially prepared serum or plasma; (c) the induction and expression of "adipocyte-specific" genes such as aP2, adipoprotein, and lipoprotein lipase appears to be a result of the particular agents used to induce adipogenesis, as well as the differentiation status of the cell; and (d) since the differentiation process mediated by sodium butyrate does not require prior growth arrest, this system can be used to distinguish the molecular and biochemical events associated with terminal differentiation from those events associated with growth arrest but not differentiation.

Terminal adipocyte differentiation was determined in these studies by comparing the morphological and molecular phenotype of cells after each of the various treatments to that exhibited by Swiss 3T3 cells terminally differentiated by one well established method (i.e. confluent cultures treated with MIX, insulin, and dexamethasone). The morphological and molecular properties analyzed here were representative of many properties previously shown to be induced in preadipocyte cells upon conversion to mature adipocytes. For example, when confluent, growth-arrested 3T3-L1 cells are treated with adipogenic agents (i.e. MIX, insulin, and dexamethasone) they accumulate triglyceride, acquire the morphological appearance of mature adipocytes, and exhibit high levels of fat cell-specific enzymes including lipoprotein lipase, glycerol-3-phosphate dehydrogenase, ATP-citrate lyase, acetyl-CoA carboxylase, and fatty acid synthetase (44, 53-56). We analyzed (and quantitated) lipid accumulation and adipocyte morphology by histochemical examination following Oil Red O and Nile Red staining. Adipocyte-related enzyme induction was assayed at the molecular level by monitoring the induction and maintenance of high levels of lipoprotein lipase mRNA. Finally, terminal adipocyte conversion was confirmed by analysis of the induction and continued expression of two transcripts found exclusively in 3T3 L1 cells induced to terminally differentiate into adipocytes, adipoprotein, and adipocyte P2 (21, 37-42). Since Swiss 3T3 cells treated with either sodium butyrate and dexamethasone or sodium butyrate and insulin exhibited morphological and molecular phenotypes consistent with that of the positive control cultures induced to differentiate by well established methods, we concluded that either of these combinations of agents, when added to subconfluent cultures of actively growing Swiss 3T3 cells, can mediate the differentiation of these cells into mature adipocytes.

It should be noted that all of these studies were performed with a nonclonal "wild-type" Swiss 3T3 cell line. As a result, it is not likely that our findings are restricted to only a specific subclone of cells which is highly susceptible to adipocyte conversion. While it is true that in the experiment shown in Table I, the spontaneous conversion of confluent cultures was fairly high (25%), it was not true of different batches of the same Swiss 3T3 cells used in the dose-response and time-course experiments shown in Figs. 2 and 3 and the adipocytic agent removal experiment shown in Table II. In both of these cases, the untreated controls converted at a frequency of 10% or less. Nevertheless, this variation in extent of spontaneous adipocyte conversion is not without precedent. In a classic study performed with this same Swiss 3T3 cell line, Green and Kehinde (13, 14, 16) took 20 well-isolated "random" clones of Swiss 3T3 cells, grew each in mass culture, then tested them for ability to convert to adipocytes. The results show that the 20 clones, only one was totally unable to give rise to adipose cells. The other 19 clones exhibited a wide range in frequency of conversion extending from very low, to moderate, to high. In fact, one clone was obtained which exhibited a frequency even higher than the 3T3-L1 clone. Moreover, even the one clone which was thought to be unable to convert was, upon further subculture, found to give rise to subclones which again exhibited a wide range of conversion frequencies. One of the factors which is thought to contribute to this variation is fetal calf serum. Green and coworkers (57-59) have attempted to suppress spontaneous differentiation by maintaining cells in the presence of mixtures of cat and calf sera, and Smith et al. (60) have shown that induction of differentiation is dependent upon the levels of insulin-like growth factor 1 and insulin in sera. It is possible that the batches of fetal calf serum used to maintain our cells contained different amounts of insulin-like growth factor 1, and as a result, we obtained a variation in the frequency of spontaneous differentiation once the cultures reached confluence. However, in all of our studies, the frequency of conversion of subconfluent cells was low (10% or less) and increased significantly (5-6-fold) only following treatment with either sodium butyrate and insulin or sodium butyrate and dexamethasone.

Interestingly, while the sodium butyrate/dexamethasone or sodium butyrate/insulin treatment induced morphological characteristics strikingly similar to those reported in cells differentiated by several other previously established methods, cells differentiated by this method did exhibit some differences at the molecular level. For example, despite the fact that both combinations were equally efficient at inducing adipogenesis at the morphological level, adipoprotein mRNA was induced only in cells treated with sodium butyrate plus dexamethasone, and aP2, while induced by both treatments was expressed at considerably higher levels following the addition of sodium butyrate/dexamethasone. Moreover, treatment of cells with any of these agents individually, a regime which did not lead to the accumulation of high levels of intracellular lipid, induced the expression of aP2 and lipoprotein lipase. This apparent inconsistency can be explained by a number of factors. First, our studies employed the RNase-protection assay, an extremely sensitive method for RNA analysis. It is possible that our ability to detect these transcripts in the
absence of terminal differentiation simply reflects the greater sensitivity of our assay. However, if this were the case, we would expect that those treatments which lead to adipogenesis should induce higher levels of aP2 and LPL. We did not observe this. Another possible explanation could be that these genes are early events in the process of adipocyte differentiation. It is possible that any of these agents could initiate adipogenesis but not maintain it. Our previous results suggest that this is certainly the case with sodium butyrate (33). We do not know if insulin or dexamethasone would exhibit the same property. A third possibility is that this method differed from those previously reported in that sodium butyrate was employed to induce adipocyte differentiation in cells which had not been previously growth-arrested or contact-inhibited. It is possible that the expression of these genes is influenced by either the sodium butyrate itself or the growth status of the cells at the time of treatment. In fact, we found that the level of LPL expression in nontreated, confluent (growth-arrested) cultures was higher than that detected in subconfluent cultures treated with either sodium butyrate plus insulin or sodium butyrate plus dexamethasone (data not shown). While any or all of these explanations may account for our observations, the results of our molecular analysis clearly show that the expression of "adipocyte-specific" genes is as much a reflection of the particular agents used to induce adipogenesis as it is of the fact that the cells are adipocytes.

In fact, the literature does contain a number of examples in which the expression of adipocyte-specific genes can be either dissociated from the adipocyte phenotype or, conversely, induced in response to treatment with individual agents which are incapable of inducing adipogenesis on their own. For example, Dani et al. (61) have shown that expression of adipsin mRNA is reduced in fat cells from ob/ob mice in vivo but is high in cultured adipocyte cell lines from these same animals. Likewise, Spiegelman and colleagues (69) have also reported that adipin expression was dramatically induced in adipose tissues from ob/ob and db/db mice; however, the expression of two other adipocyte-specific genes, glycerol-3-phosphate dehydrogenase and aP2 expression was essentially identical in both lean and obese mice. This was also true in animals in which obesity was induced by injection of monosodium glutamate. Rubin and colleagues (60) have shown that either insulin or insulin-like growth factor 1 is required for induction of aP2 and glycerol-3-phosphate dehydrogenase in 3T3-L1 preadipocytes (and appearance of the adipocyte phenotype). Yet treatment of these cells with either insulin or insulin-like growth factor 1 alone cannot induce adipocyte differentiation (59). Finally, a number of recent reports have identified glucocorticoid and cAMP-responsive cis-acting elements in the promoters of adipocyte-specific genes. Cook et al. (63) have cloned, sequenced, and analyzed 858 nucleotides of 5'-flanking region of aP2 and found two separate regions capable of conferring transcriptional responsiveness to dexamethasone and cAMP, respectively. This is consistent with our finding that aP2 mRNA could be detected in cells which were treated with dexamethasone even though they did not exhibit the adipocyte phenotype. A glucocorticoid-responsive element has also been identified in the promoter region of glycerol-3-phosphate dehydrogenase and stearyl-CoA desaturase (64). Interestingly, a glucocorticoid response-element has not been identified in the promoter of the adipsin gene (64), a finding consistent with our inability to detect adipin mRNA in glucocorticoid-treated cell samples. Superimposed upon what already appears to be a complex regulatory problem is the recent identification of both positive and negative regulatory elements in the aP2 promoter (65). It would appear, therefore, that molecular criteria such as the expression of adipocyte-specific genes is not sufficient to determine whether differentiation has occurred.

In light of the well-established antiproliferative activity of sodium butyrate (1–5) it is worthwhile to consider whether the role of this agent in the differentiation process was merely one of arresting cell growth thereby creating a situation analogous to that described previously in confluent, growth-arrested cultures. However, there are three lines of experimental evidence which suggest that this was not the case. First, our previous work showed that sodium butyrate alone induced molecular and morphological events known to be associated with adipocyte differentiation as well as with growth arrest (33). Second, dexamethasone or insulin treatment of confluent, growth-arrested cells did not induce the accumulation of high concentrations of intracellular lipid (Table I). Since treatment with both sodium butyrate and dexamethasone or sodium butyrate and insulin did, it would appear that sodium butyrate is required to achieve complete adipogenesis. Third, terminal differentiation occurred even when cells were first treated, during active growth, with dexamethasone or insulin, and then treated 24 h later with sodium butyrate (data not shown) This suggests that each of these agents induces an independent series of events which mediate the conversion of these cells into terminal adipocytes.

While we are convinced that sodium butyrate plays more than merely an antiproliferative role in the terminal differentiation process, we still do not know precisely what that role might be. It is interesting to note that sodium butyrate arrests cells at a point in G1 which is both temporally and molecularly distinct from that induced by either growth to high density or growth factor deprivation (4, 33, 66, 67). In another well-characterized adipocyte model system the mouse 3T3 T-mesenchymal stem cell system (68–71), the cells are first arrested at a distinct stage of G1, called G1n, by growth in specially fractionated human plasma. Like sodium butyrate arrest, G1n differs from the G1 arrest state induced by growth to high density or growth factor deprivation, 3T3 cells in this state are preadipocytes which can be induced to either differentiates (when treated with heparinized human plasma or 30% serum plus insulin) or to reenter the cell cycle (when treated with MIX) (70, 71). Similarly, sodium butyrate-treated cells also arrest in a distinct stage of G1 (33), exhibit properties which might suggest that they can differentiate into adipocytes, and can be induced to either terminally differentiate (by addition of either dexamethasone or insulin) or proliferate (by removal of the sodium butyrate). It is therefore possible that sodium butyrate treatment of actively proliferating cells induces a state similar to the preadipocyte G1p state described by Scott (68–71). However, it should be noted that the sodium butyrate-treated cells appear to be similar but not identical to cells in G1p. The most obvious difference between these two systems is that although sodium butyrate-induced growth arrest is reversible, treatment of sodium butyrate-arrested cells with MIX does not lead to active proliferation, unlike the 3T3 T-mesenchymal T-cell system. Nevertheless, the considerable similarity between these two states suggests that sodium butyrate and G1p arrest induce similar events which predispose a Swiss 3T3 cell to respond to terminal differentiation agents. However, the nature of the events common to these treatments awaits further investigation.

It would appear that the sodium butyrate-treated Swiss 3T3 cell represents an in vitro system representative of a physiological state at which growth arrest and differentiation are coupled. This is important because the processes which cause cells to simultaneously lose the ability to traverse the cell...
cycle and gain the ability to undergo terminal differentiation represent important regulatory events in the control of cell growth in vivo. Obviously, an *in vitro* model system which permits one to clearly distinguish those processes associated with terminal differentiation from those associated with loss of growth potential would greatly facilitate the study of these regulatory events. We believe that the treatment of actively proliferating Swiss 3T3 cells with sodium butyrate followed by either insulin or dexamethasone constitutes just such a system. In addition, it has the added advantage over systems such as the mouse 3T3 T-mesenchyme system in that it can be consistently and easily reproduced using commercially available reagents. By analysis at the molecular level of individual events modulated exclusively by each reagent, we anticipate that we will ultimately be able to elucidate at least some of the details involved in the complex process of growth regulation.

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