Regulation of Glucose-6-phosphatase Gene Expression in Cultured Hepatocytes and H4IE Cells by Short-chain Fatty Acids

ROLE OF HEPATIC NUCLEAR FACTOR-4α*

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Mechanisms underlying dietary nutrient regulation of glucose-6-phosphatase (Glc-6-Pase) gene expression are not well understood. Here we investigated the effects of short-chain fatty acids on the expression of this gene in primary cultures of rat hepatocytes and H4IE hepatoma cells. Propionate, butyrate, valerate, and caproate induced severalfold increases in the expression of Glc-6-Pase mRNA. In reporter gene assays, propionate, valerate, caproate, and also octanoate increased Glc-6-Pase promoter activity by 616-fold. Butyrate, by itself, had little or no effect on promoter activity, but it induced a robust increase (45-fold) in promoter activity in cells co-transfected with a plasmid expressing the transcription factor HNF-4α (α isoforms of hepatic nuclear factor 4). HNF-4α also enhanced promoter activity induced by other short-chain fatty acids. A dominant negative form of HNF-4α abrogated the fatty acid-induced promoter activity, a finding that accentuates a role for HNF-4α in the transcription process studied here. In cells transfected with HNF-4α, short-chain fatty acids and trichostatin A, an inhibitor of histone deacetylase, synergistically enhanced promoter activity, suggesting that hyperacetylation of histones is an important component of the transactivation of the Glc-6-Pase gene promoter by HNF-4α. Region -751/ -466 of this promoter contains seven putative HNF-4α-binding motifs. Binding of HNF-4α to this region was confirmed by electrophoretic mobility shift and chromatin immunoprecipitation assays, indicating that HNF-4α is recruited to the Glc-6-Pase gene promoter during short-chain fatty acid-induced transcription from this promoter.

Glucose-6-phosphatase (Glc-6-Pase) is expressed in the liver, kidney, and small intestine and catalyzes the hydrolysis of glucose 6-phosphate during gluconeogenesis and glycogenolysis. Hepatic Glc-6-Pase is a multicomponent complex located in the endoplasmic reticulum and consists of at least five different proteins (2, 3) that include three transporters termed T1, T2, and T3, the catalytic unit, and a stabilizer protein (2, 4–6). Hormones and nutrients such as glucose and fatty acids, which are elevated in poorly controlled diabetes, profoundly modulate the expression of the gene for the catalytic unit (Glc-6-Pase) (7–9). Whereas glucose strongly activates expression of this gene, the effect of fatty acids is quite complex and appears to depend on the chain length and state of saturation of the fatty acid (1, 9). For example, polyunsaturated fatty acids inhibit, while long-chain saturated fatty acids induce expression of this gene (1, 9). However, virtually nothing is known as to whether short-chain fatty acids modulate the expression of the Glc-6-Pase gene or any genes encoding gluconeogenic enzymes.

Although the role of nutrients as regulators of gene expression is well documented (10–13), the molecular mechanisms by which nutrients, especially fatty acids, modulate gene expression remain a challenge (11, 14). Stimulation of Glc-6-Pase gene transcription by long-chain saturated fatty acids has been attributed to stabilization of the mRNA (1). However, the effects of saturated fatty acids may also involve gene transcription, although this has not been tested. Modulating effects of short-chain fatty acids, if any, have also not been explored. Here we have studied the effect of short-chain fatty acids on Glc-6-Pase gene expression because, like long-chain fatty acids, short-chain fatty acids can also play an important role in energy generation. Using primary cultures of rat hepatocytes and the hepatoma cell line H4IE, we show that short-chain fatty acids induce Glc-6-Pase gene transcription and that this induction occurs via recruitment of the transcription factor HNF-4α to the Glc-6-Pase promoter.

EXPERIMENTAL PROCEDURES

Cell Culture and Transient Transfections—Rat hepatocytes were isolated as described previously (15). Primary cultures were maintained in RPMI 1640 medium containing 5 mM glucose (15). H4IE cells were cultured in 6-well plates at 37 °C in Dulbecco’s modified Eagle’s medium/F-12 (Invitrogen) containing 10% fetal bovine serum and 5% calf serum. Cells were transfected with 1 μg of a −751/-66 proximal portion of the mouse Glc-6-Pase gene promoter linked to luciferase reporter gene using FuGENE 6 transfection reagent (Roche Applied Science) at a 1:1 ratio of FuGENE 6 to DNA according to the manufacturer’s instructions. Because we found that various fatty acids induced reporter gene expression in conventional reporter constructs (e.g. Renilla and β-galactosidase) used for normalization in transfection experiments, making it impractical to use such plasmids for this purpose in our experiments, we normalized luciferase activity to the protein content of each sample. In co-transfection experiments, 1 μg of luciferase reporter plasmid was co-transfected with 100 ng of CDNA expression plasmid, and the total amount of DNA was adjusted, if necessary, by adding the empty plasmid. Cells were harvested 24 h after addition of
fatty acids, washed twice in 1× phosphate-buffered saline (PBS), and then lysed with 300 μl of Cell Culture Lysis Buffer (Promega, Madison, WI). The lysates were centrifuged at 13,000 × g for 5 min, and 10 μl of the supernatant solution were assayed for luciferase activity. Protein concentrations were determined by the Lowry method using a Bio-Rad assay kit according to the manufacturer’s protocol. Each transfection was performed in duplicate at least three times.

Expression Plasmids—Expression plasmids harboring genes for wild-type and dominant negative HNF-4α were obtained from Dr. Todd Leff (University of Michigan, Ann Arbor, MI). cDNA for Glec-6-Pase was obtained from Dr. Rebecca Taub (University of Pennsylvania, Philadelphia, PA). cDNA for glucose-6-phosphate translocase was obtained by PCR amplification using the following primers: 5′-ACACAGGACATCCACTGCTG-3′ and 5′-CTCTGCTGAGGACTCTGAGA-3′ (GenBank™ accession number NM_031588). The resulting cDNA was subcloned into pCMV-Tag 2A (Stratagene, La Jolla, CA), and the nucleotide sequence was verified by DNA sequencing.

Northern Blotting—TRIZOL reagent (Invitrogen) was used to isolate total RNA according to the manufacturer’s protocol. The isolated RNA was assessed for purity by the 260/280 nm absorbancy ratios. Electrophoresis of the RNA (20 μg) was performed on a 1.2% formaldehyde-denatured agarose gel in 1× MOPS running buffer. After electrophoresis, RNA was transferred to a Hybond-N+ membrane (Amersham Biosciences) and prehybridized for 4 h at 55°C in Church buffer (0.5 M Na2HPO4, pH 7.2, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin). Hybridization with the 32P-labeled probe that had been ligated into pCMV-Tag 2A (Stratagene, La Jolla, CA), and the nucleotide sequence was verified by DNA sequencing. Next, the membrane was washed twice in ice-cold PBS and collected by scraping in 1 ml of 1% SDS phosphate buffer, pH 7.0, 7% SDS, 1 mM EDTA, and 1% bovine serum albumin. Formaldehyde was then neutralized at room temperature (5 min) with 125 mM of glycine and the samples were resuspended in 1 ml of buffer (1% SDS, 10 mM EDTA, 50 mM KCl, 0.5 mM dithiothreitol, 1.5 mM PMSF, 0.3 mM Na3VO4, 5 mM NaF, 1× protease inhibitor mixture (Roche Applied Science), and 0.05% Nonidet P-40), incubated on ice for 10 min, and centrifuged at 14,000 g for 10 min. The supernatant fraction was frozen in aliquots and stored at −80°C.

Electrophoretic Mobility Shift Assays (EMSAs)—Oligonucleotide probes were end-labeled with T4 polynucleotide kinase (New England BioLabs, Beverly, MA). Nuclear extracts (5 or 10 μg of protein) from H4IE cells were incubated for 15 min at room temperature in the presence of 2 μg of poly(dI-dC) in a reaction mixture containing 25 mM HEPES (pH 7.9), 60 mM KC1, 2.5 mM MgCl2, 0.5 mM EDTA, 0.5 mM Na3VO4, 5 mM NaF, 1× mixture of protease inhibitor (Roche Applied Science), 0.5 mM PMSF, 0.1% Nonidet P-40, and 25% glycerol and incubated on ice for 30 min. After centrifugation at 14,000 × g, the supernatant fraction was frozen in aliquots and stored at −80°C.

Hepatic Glucose-6-phosphatase Gene Expression—In Northern blotting assays to assess the effects of short-chain fatty acids (acetate, propionate, butyrate, valerate, and caproate) on steady-state levels of Glec-6-Pase mRNA, acetate had no effect, whereas all other short-chain fatty acids tested induced robust increases in Glec-6-Pase mRNA levels in primary cultures of rat hepatocytes (Fig. 1). This effect was gene-specific because mRNA levels for glucose-6-phosphate translocase, which is associated with the Glec-6-Pase complex, was not affected by the various fatty acids tested (Fig. 1). Next we wondered whether short-chain fatty acids might affect the expression of another gene encoding a key gluconeogenic enzyme. In this regard, we assessed the effect of the short-chain fatty
fatty acids. At these concentrations, the short-chain fatty acids, each added at 2.5 mM, induced 15–17-fold increases in the activity of the PEPCK promoter (Fig. 2A). Taken together with the data on the kinetics of induction of this promoter by butyrate, the mechanism of induction of this promoter by butyrate may prove to be more complex than that by other short-chain fatty acids. We did not observe cell death in our studies with butyrate as assessed by trypan blue dye exclusion; therefore, apoptosis reported in PLC/PRF/5 hepatoma cells cultured for 72 h with butyrate (23) was not manifest in our experiments.

To identify the fatty acid-responsive region(s) within the Glc-6-Pase promoter, a series of deletions in the −575/+66-LUC construct were created by exonuclease digestion and by PCR. The constructs were then used in transient transfection assays (Fig. 3) with caproate as the test fatty acid. Sequence deletion in the −751/−553 segment resulted in induced promoter activity in both basal (about 4-fold) and fatty acid-stimulated (up to 18-fold) conditions. Deletions in the −466/−276 segment provoked a progressive decline in promoter activity, but beyond −276 bp there was no major effect of the fatty acid. Thus, it is apparent that the −751/−276 region of the promoter accounted for most of the robust transcriptional response to the fatty acid. Similar results were obtained with valerate. Our interest in deciphering the identity of the cis-positive elements that are involved in the transcriptional activation of Glc-6-Pase by the short-chain fatty acids was stimulated by the report (9) that overexpression of the transcription factor HNF-4α can transactivate the rat Glc-6-Pase promoter in HeLa cells, which normally do not express this gene. It is known that HNF-4α binds DNA as homodimers to the direct repeat of the nucleotide consensus sequence 5’-AGGTCA-3’ separated by one (DR1) or two base pairs (DR2) (24). Inspection of the Glc-6-Pase promoter using the TRANSFAC data base (25) shows seven DR elements for hormonal regulation of this gene (20–22), the promoter. We then used the luciferase reporter gene assay to monitor transcription from the transfected promoter. For these experiments, H4IIE cells were transfected with a 2.5 mM concentration of each fatty acid with the exception of caproate and valerate, which were used at 5 mM. After 4 h, total RNA was isolated, and mRNA was measured by Northern blot analysis. The same blot was reprobed for glucose-6-phosphate translocase (G6PT) and PEPCK mRNAs. The blot shown is representative of three experiments. The picture of ethidium bromide-stained gel (bottom panel) depicting 18 and 28 S rRNAs is shown to indicate sample loading.

Transcriptional Activation of Glc-6-Pase by Short-chain Fatty Acids Requires DNA Sequence Elements Located in the Non-coding Region—The data in Fig. 1 demonstrate that short-chain fatty acids induce Glc-6-Pase expression from the endogenous promoter. We then used the luciferase reporter gene assay to monitor transcription from the transfected promoter. For these experiments, H4IIE cells were transfected with a −751/+66 bp fragment of the Glc-6-Pase promoter linked to the luciferase reporter gene (−751/+66-LUC) (16), and the transcriptional activity of this promoter construct in the presence of various short-chain fatty acids was then measured. In dose-response pilot experiments, valerate was the most potent, producing maximal response at 1 mM, while propionate and caproate induced maximal promoter activity at 2.5 mM (data not shown). Therefore, subsequent experiments were carried out with a 2.5 mM concentration of each fatty acid with the exception of valerate, which was used at 1 mM. At these concentrations, caproate and valerate induced a 5–6-fold increase in promoter activity (Fig. 2A); propionate had a much lesser effect (about 2-fold), while acetate and butyrate had little or no effect (Fig. 2A).

We also assessed the effect of the short-chain fatty acids on transcription from the PEPCK gene promoter, a promoter that responds also to gluconeogenic stimuli (17–19). When the H4IIE cells were transfected with a −490 bp proximal fragment of the PEPCK gene promoter that has all the necessary elements for hormonal regulation of this gene (20–22), the short-chain fatty acids elicited substantial increases in PEPCK gene promoter activity, the most potent being butyrate and valerate, which induced 16–17-fold increases in the activity of the promoter (Fig. 2B). Taken together with the data on the mRNA levels for both PEPCK and Glc-6-Pase (see Fig. 1), these results suggest a concerted response to the inductive effect of these short-chain fatty acids by at least two genes encoding two different gluconeogenic enzymes.

The little or no effect of butyrate on the Glc-6-Pase promoter activity was rather surprising considering that this fatty acid induced robust increases not only in Glc-6-Pase mRNA level (see Fig. 1, top panel) but also in PEPCK mRNA levels and PEPCK gene promoter activity (see Fig. 2B). However, it is important to note that in other experiments described in Fig. 5 (A and B), the inductive effect of butyrate on Glc-6-Pase promoter activity was evident when the cells were co-transfected with expression plasmid for HNF-4α, suggesting that the mechanism of induction of this promoter by butyrate may be more complex than that by other short-chain fatty acids. We did not observe cell death in our studies with butyrate as assessed by trypan blue dye exclusion; therefore, apoptosis reported in PLC/PRF/5 hepatoma cells cultured for 72 h with butyrate (23) was not manifest in our experiments.

To verify whether these sites actually bind HNF-4, EMSAs were performed. For these assays, we used two labeled probes corresponding to nucleotides −751/−686 (Fig. 4, B, C, and D) and −462/−382 (Fig. 4E) in the Glc-6-Pase gene promoter. The −751/−686 probe contains sites 1 and 2, while the −462/−382 probe contains sites 4 and 5 in Fig. 2A. Three or four protein-DNA complexes (see arrows in Fig. 4) were detected with both probes; with the −462/−382 probe the signals were more intense than with the −751/−686 probe, suggesting that binding sites in this probe may have higher affinity for HNF-4 than sites in the −751/−686 probe. In both cases, unlabeled probes competitively displaced these complexes as expected. In experiments with the −751/−686 probe, an oligonucleotide (CCAGCGCCATGTGCTATT) (−749/−726) that contained the sequence (underlined) identified as site 1 in Fig. 4A was an effective competitor against the formation of these complexes (Fig. 4C, lanes 4 and 5 versus lanes 2 and 3). Against this probe, an oligonucleotide (TCCAGGGACAAAGCCCTAC) (−459/−439) that contained the sequence (underlined) identified as site 4 in Fig. 2A was not as effective a competitor as the −749/−726 oligonucleotide (Fig. 4D, lanes 4 and 5 versus lanes 2 and 3), although some displacement was noticeable. This was not surprising since site 4 is absent in the −751/−686 probe. However, in experiments conducted with the −462/−382 probe (Fig. 4E), this oligonucleotide (i.e. TCCAGGGACAAAGCCCTAC) (−459/−439) was a much better competitor against this probe (Fig. 4E, lanes 6 and 7 versus lanes 2 and 3) than against the −751/−686 probe (see Fig. 4D). This is consistent with the fact that site 4 is absent in the −751/−686 probe.
Because the two probes used for these assays contain four of the seven DR motifs in the Glc-6-Pase gene promoter, we interpret these oligonucleotide competition data to mean that one or more of these sites can bind HNF-4α. To confirm this notion, anti-HNF-4α antibodies were used in the gel mobility shift assays. Although no clearly defined supershifted band was discernible, band intensity of the slowest moving band decreased in the presence of anti-HNF-4α antibodies (Fig. 4, B and E, compare lane 2 with lane 4 and lane 3 with lane 5 for both panels), a phenomenon usually indicating that the antibodies may be preventing DNA binding or that proteins binding to sequences adjacent to the transcription factor-binding site(s) interfere with binding of transcription factor to the probe used (26). These results are consistent with the interpretation that HNF-4α is contained in the protein component of this complex.

If HNF-4α were relevant for short-chain fatty acid-induced regulation of Glc-6-Pase gene expression, then transfection of plasmids harboring the gene for HNF-4α would be expected to increase transcription from the Glc-6-Pase gene promoter. Conversely, transfection of dominant negative HNF-4α should interfere with transcription from this promoter. Both experimental approaches were utilized in this study. First, using the wild-type promoter (−751/+66-LUC) and a truncated version (−553/+66-LUC) that exhibited the highest promoter activity as seen in Fig. 3, co-transfection with an expression plasmid for HNF-4α provoked robust increases in promoter activity in response to various short-chain fatty acids as well as to octanoate, a medium-chain fatty acid (Fig. 5, A and B). With the wild-type promoter construct (−751/+66-LUC), transcriptional response to the addition of HNF-4α was 6–16-fold (Fig. 5A), whereas with the −553/+66-LUC promoter construct, the response was substantially higher (40–100-fold) (Fig. 5B). Even butyrate, which by itself exerted very little effect on the Glc-6-Pase promoter activity (see Fig. 2A), robustly increased the activity of both promoter constructs in the presence of HNF-4α.

**Fig. 2.** Short-chain fatty acids induce increased transcription from the Glc-6-Pase and PEPCK gene promoters. H4IIE cells were transfected with −751/+66-LUC (A) or PEPCK −490-LUC (B). After 24 h, the various fatty acids (valerate, 1 mM; all others, 2.5 mM) were added. The cells were harvested for luciferase assay 24 h later. Luciferase activity was assessed as described under “Experimental Procedures.” Promoter activity was normalized to the protein content of the samples and is expressed as -fold stimulation over control (no fatty acid). The results shown are the means ± S.E. of four different experiments.

**Fig. 3.** Activities of Glc-6-Pase gene promoter constructs. H4IIE cells were transfected with the various Glc-6-Pase promoter fragments indicated. After 24 h, cells were treated with 2.5 mM caproate and harvested for luciferase assay 24 h later as described under “Experimental Procedures.” The results are the means ± S.E. of four different experiments. Promoter activity was normalized to the protein content of the sample; the activity of each promoter construct is expressed relative to the activity of the wild-type promoter in the absence of fatty acid (Con).
Second, when the −751/+66-LUC promoter construct was co-transfected with a plasmid harboring the dominant negative form of HNF-4α (27), the promoter activity-inducing effect of valerate was diminished to 1.2-fold (Fig. 5C) compared with 6-fold seen in cells not transfected with the dominant negative plasmid (see Fig. 5A). Although not shown here, this dominant negative effect was also observed with other short-chain fatty acids. These results confirm that the transcriptional response indeed requires HNF-4α. Taken together with the data from gel mobility shift assays, these results indicate that HNF-4α is involved in the short-chain fatty acid-induced transcription from the Glc-6-Pase gene promoter.
Fig. 6. Detection of HNF-4α in H4IIE cells by Western blotting. H4IIE cells were transfected with (lanes 1 and 2) or without (lanes 3 and 4) 100 ng of an expression plasmid harboring the gene for the transcription factor HNF-4α and then treated with or without valerate (1 mM) for 16 h. The cells were then washed with cold 1× PBS and lysed; the supernatant and nuclear fractions were separated as indicated under “Experimental Procedures.” Twenty micrograms of nuclear extract proteins were separated onto a 7.5% SDS-polyacrylamide gel and analyzed by Western blotting (A). Staining the membrane with fast green (B) was used to assess loading irregularities.

On Western blots, HNF-4α was clearly detected in nuclear extracts (Fig. 6A) from cells transfected with an expression plasmid harboring the gene for HNF-4α (lanes 1 and 2) but was barely detectable in non-transfected (control) cells (lanes 3 and 4). Based on fast green staining of the polyvinylidene difluoride membranes (used as loading controls, Fig. 6B), there was no dramatic difference in the concentration of HNF-4α in cells treated with the short-chain fatty acid valerate compared with cells that were not treated with the fatty acid.

HNF-4α Is Recruited to the Glc-6-Pase Promoter—To provide evidence of direct interaction of HNF-4α with the Glc-6-Pase gene under conditions of short-chain fatty acid activation in vivo, a simple and most direct approach is to stimulate the cells with the short-chain fatty acids and then perform chromatin immunoprecipitation assays. Therefore, we stimulated H4IIE cells, which were transfected with the −750/+66-LUC promoter fragment, with 2.5 mM valerate for 16 h and immunoprecipitated the DNA-protein complexes with anti-HNF-4α antibody. The binding of HNF-4α to its putative binding site corresponding to sites 4 and 5 in Fig. 4A was analyzed by PCR and visualized on an agarose gel (Fig. 7). A PCR signal is an indication of the presence of HNF-4α in a complex binding to its putative binding site. As shown in Fig. 7, the presence of HNF-4α was detected only in valerate-treated cells that were immunoprecipitated with anti-HNF-4α antibody. The binding is specific since negative controls using samples in which the antibody was omitted did not show a signal. These results indicate that HNF-4α binds to the Glc-6-Pase gene promoter in vivo and that this binding is enhanced in response to valerate.

Histone Acetylation Is Necessary but Not Sufficient to Transactivate the Glc-6-Pase Gene—Of the fatty acids studied here, the best known for exhibiting the most pleiotropic effects on genes is perhaps butyrate (28–32). These effects have largely been attributed to inhibition of histone deacetylase(s) (HDAC) (29–33). To determine whether histone acetylation plays a role in the short-chain fatty acid-induced transcription from the Glc-6-Pase gene promoter, two series of experiments were carried out. In the first series, we examined to what extent histones H3 and H4, as prototype histones, were acetylated in primary cultures of rat hepatocytes treated with short-chain fatty acids. Histone (H3 and H4) acetylation was monitored at 2 and 4 h after addition of fatty acids to the cultures. At 2 h, acetate, butyrate, and valerate had no effect on the levels of acetylated histone H4, whereas propionate and caproate induced 3-fold increases in this parameter (Fig. 8, left panel). At 4 h, butyrate- and valerate-induced acetylation of histone H4 increased by 4-fold, whereas acetylation caused by acetate, propionate, and caproate returned to basal levels (Fig. 8, right panel). The same pattern of acetylation was observed with histone H3. As noted in Fig. 1, robust levels of Glc-6-Pase mRNA were attained within the time frame studied in Fig. 8. Therefore, we surmised that acetylation of histones could be involved in the induction of Glc-6-Pase gene expression by short-chain fatty acids.

In the second series of experiments, we carried out transfections in the presence of trichostatin A (TSA), a well characterized inhibitor of mammalian HDACs (34). At 10 and 100 ng of TSA/ml, TSA increased Glc-6-Pase gene promoter activity by 3–5-fold (Fig. 9A). In cells transfected with an expression plasmid harboring the gene for the transcription factor HNF-4α, TSA-induced promoter activity was about 45-fold at 100 ng of TSA. Furthermore, in cells treated with TSA and a short-chain fatty acid, transfection of HNF-4α elevated the transcriptional response to levels far exceeding those previously observed for either TSA alone or fatty acid alone (Fig. 9B). Because TSA and the fatty acids used here inhibited HDAC (29, 34, 35), we interpret these findings to mean that hyperacetylation of histones or other proteins is an important component of the transactivation of the Glc-6-Pase gene promoter by HNF-4α. In experiments in which the Glc-6-Pase promoter construct was co-transfected with a plasmid harboring the dominant negative form of HNF-4α (27) as in Fig. 5C, the promoter-inducing effect of the fatty acids was abrogated whether or not TSA was present in the cultures. Taken together, these results strongly suggest that histone acetylation is necessary but not sufficient to account for short-chain fatty acid-induced transactivation of the Glc-6-Pase gene.
Fatty acids have been reported to modulate several genes encoding enzymes of lipogenic and gluconeogenic significance (11, 14), but suggested mechanisms of this modulation are rather complex (14). With respect to the Glc-6-Pase gene, polyunsaturated fatty acids inhibit, while long-chain saturated fatty acids stimulate expression. The effects of short-chain fatty acids have not been studied. Here we have evaluated the ability of five short-chain fatty acids (acetate, propionate, butyrate, valerate, and caproate) to modulate Glc-6-Pase gene expression in cultured hepatocytes and hepatoma cells. Potential molecular mechanisms for such modulation were also explored. With the exception of acetate, these fatty acids increased Glc-6-Pase mRNA levels as well as transcription from the exogenous promoter as measured by transient transfection assays. The transcription factor HNF-4α was necessary to achieve butyrate-induced increase in Glc-6-Pase promoter activity. The short-chain fatty acids also increased the expression of PEPCK, an enzyme catalyzing the first committed step in the pathway of gluconeogenesis from lactate and amino acids.

In recent years, it has become evident that acetylation of histones is associated with transcribed regions in chromatin (36). Proteins with histone acetyltransferase activity are co-activators, while proteins with HDAC activity behave as corepressors (36). HDAC activity is often associated in protein complexes with NcoR (nuclear co-repressors) and SMRT (silencing mediator of receptor transcription) that mediate nuclear receptor repression (37–39). Inhibitors such as TSA and trapoxin relieve HDAC-induced transcriptional repression, indicating an essential role for HDAC in this process (35). Because butyrate and possibly other short-chain fatty acids inhibit HDAC (29, 34, 35), it was of interest to examine whether the transcriptional effects of short-chain fatty acids on the Glc-6-Pase gene promoter could be attributed to their ability to increase histone acetylation. Our study demonstrates that these fatty acids increased the level of acetylation of histones H3 and H4, which were monitored as prototype histones. A role for histone acetylation was further evidenced by the fact that when H4IIE cells were incubated with TSA, there was potentiation of the effects of the short-chain fatty acids on the Glc-6-Pase promoter activity. It is interesting to note that acetate, a substrate for histone acetylation, was not effective in stimulating Glc-6-Pase promoter activity.

If acetylation of histone is required to transactivate the Glc-6-Pase promoter, then a complex must be assembled in order for this activation to take place; therefore some transcription factor(s) must be recruited to the Glc-6-Pase promoter in response to the short-chain fatty acids. Sp1 is often implicated in transcriptional repression (40) or activation (23, 41) by butyrate, a classical HDAC inhibitor. Also it has been reported that Sp1 is essential for basal activity of the Glc-6-Pase gene promoter (42). However, it seems unlikely that this transcription factor is involved in the fatty acid effects on the Glc-6-Pase gene promoter reported here since overexpression of Sp1 in H4IIE cells did not influence the response of the Glc-6-Pase promoter to the various short-chain fatty acids. Instead we found that the transcription factor HNF-4α potently modulated expression from the Glc-6-Pase gene promoter. That HNF-4α was involved was further supported by an experiment in which overexpression of a dominant negative form of HNF-4α that contains a defective DNA-binding domain but is capable of homodimerization with endogenous HNF-4α (27) prevented the stimulatory effects of all the short-chain fatty acids. The Glc-6-Pase gene promoter used in these studies contains putative binding motifs for HNF-4α. We demonstrated binding of HNF-4α to this promoter in gel mobility shift and chromatin immunoprecipitation assays.

HNF-4α belongs to the steroid superfamily of transcription factors and is abundantly expressed in the liver, the intestine, and the kidney (43, 44). Two of these tissues are major sites for gluconeogenesis in mammals. Several observations suggest that in combination with other liver-specific or ubiquitous transcription factors HNF-4α plays an important role in liver-specific gene expression (45–48). For example, HNF-4α has been implicated in modulation of the L-type pyruvate kinase gene by glucose (49). More recently, removal of HNF-4α from its consensus-binding site on DNA has been demonstrated as the mechanism by which polyunsaturated fatty acid suppresses the activity of the Glc-6-Pase gene (9). The ability of HNF-4α to transactivate its target genes is greatly potentiated by the presence of inhibitors of HDAC (43, 50). In this regard, it is interesting to note that in H4IIE cells overexpressing HNF-4α, TSA tremendously increased the activity of the Glc-6-Pase gene.

FIG. 9. Trichostatin A and HNF-4α synergistically stimulate transcription from the Glc-6-Pase gene promoter. In A, H4IIE cells were transfected with −751/+66-LUC alone or with −751/+66-LUC and 100 ng of an HNF-4α expression plasmid. After 24 h, some cultures were treated with the indicated concentrations of TSA, and luciferase activity was measured 24 h later. In B, cells were treated with 100 ng of TSA/ml of culture and a 1 mM concentration of the indicated fatty acids 24 h after transfection. After an additional 24 h, the cells were harvested and processed for luciferase activity. Promoter activity was normalized to the protein content of the samples. The results are means ± S.E. of three different experiments.

DISCUSSION

If acetylation of histone is required to transactivate the Glc-6-Pase promoter, then a complex must be assembled in order for this activation to take place; therefore some transcription factor(s) must be recruited to the Glc-6-Pase promoter in response to the short-chain fatty acids. Sp1 is often implicated in transcriptional repression (40) or activation (23, 41) by butyrate, a classical HDAC inhibitor. Also it has been reported that Sp1 is essential for basal activity of the Glc-6-Pase gene promoter (42). However, it seems unlikely that this transcription factor is involved in the fatty acid effects on the Glc-6-Pase gene promoter reported here since overexpression of Sp1 in H4IIE cells did not influence the response of the Glc-6-Pase promoter to the various short-chain fatty acids. Instead we found that the transcription factor HNF-4α potently modulated expression from the Glc-6-Pase gene promoter. That HNF-4α was involved was further supported by an experiment in which overexpression of a dominant negative form of HNF-4α that contains a defective DNA-binding domain but is capable of homodimerization with endogenous HNF-4α (27) prevented the stimulatory effects of all the short-chain fatty acids. The Glc-6-Pase gene promoter used in these studies contains putative binding motifs for HNF-4α. We demonstrated binding of HNF-4α to this promoter in gel mobility shift and chromatin immunoprecipitation assays.

HNF-4α belongs to the steroid superfamily of transcription factors and is abundantly expressed in the liver, the intestine, and the kidney (43, 44). Two of these tissues are major sites for gluconeogenesis in mammals. Several observations suggest that in combination with other liver-specific or ubiquitous transcription factors HNF-4α plays an important role in liver-specific gene expression (45–48). For example, HNF-4α has been implicated in modulation of the L-type pyruvate kinase gene by glucose (49). More recently, removal of HNF-4α from its consensus-binding site on DNA has been demonstrated as the mechanism by which polyunsaturated fatty acid suppresses the activity of the Glc-6-Pase gene (9). The ability of HNF-4α to transactivate its target genes is greatly potentiated by the presence of inhibitors of HDAC (43, 50). In this regard, it is interesting to note that in H4IIE cells overexpressing HNF-4α, TSA tremendously increased the activity of the Glc-6-Pase gene.

D. Massillon, unpublished.
promoter either alone or in the presence of the various short-chain fatty acids (Fig. 9). These results imply that localized alteration in chromatin structure and hyperacetylation caused by TSA treatment probably result in enhanced recruitment of the HNF-4α protein to the Glc-6-Pase promoter. Presumably HNF-4α is also acetylated under these conditions. This scenario seems plausible since in a different context (51) the acetylation of HNF-4α by cAMP-response element-binding protein (CREB)-binding protein (CBP), a phenomenon that increases its binding to DNA, appears to be necessary to retain HNF-4α in the nucleus; in the absence of such acetylation, HNF-4α is transported out into the cytoplasm (51).

Acknowledgments—We thank Amy Yi-Feei Wang for technical assistance; Dr. Richard O’Brien, Vanderbilt University, Nashville, TN, for providing us the Glc-6-Pase promoter; Dr. Rebecca Taub, University of Michigan, Ann Arbor, MI, for gifts of plasmids harboring genes for HNF-4α and dominant negative HNF-4α. We also thank Drs. Laura E. Nagy and Yumiko Kawai for critical reading of the manuscript.

REFERENCES

1. Chatelain, F., Pogorier, J. P., Minassian, C., Bruni, N., Tarpin, S., Girard, J., and Mitieux, G. (1998) Adv. Enzymol. Relat. Areas Mol. Biol. 69, 203–281
2. Patel, Y. M., Yun, J. S., Liu, J., McGrane, M. M., and Hanson, R. W. (1994) J. Biol. Chem. 269, 5619–5628
3. Liu, J., and Hanson, R. W. (1991) Mol. Cell. Biochem. 104, 89–100
4. Liu, J. S., Park, E. A., Gurney, A. L., Roesler, W. J., and Hanson, R. W. (1991) J. Biol. Chem. 266, 19095–19102
5. Hung, W. C., and Chuang, L. Y. (1999) Br. J. Cancer 80, 705–710
6. Aranda, A., and Pascual, A. (2001) Physiol. Rev. 81, 1269–1304
7. Heinemeyer, T., Chen, X., Karas, H., Kel, A. E., Kel, O. V., Liebig, I., Meinhardt, T., Reuter, I., Schachterer, F., and Wingender, E. (1999) Nucleic Acids Res. 27, 318–322
8. Carey, M., and Smale, S. T. (2000) Transcriptional Regulation in Eukaryotes: Concept, Strategies, and Techniques, pp. 266–268, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
9. Taylor, D. G., Haubenwallner, S., and Leff, T. (1996) Nucleic Acids Res. 24, 2930–2935
10. Yang, J., Kawai, Y., Hanson, R. W., and Arinne, I. J. (2001) J. Biol. Chem. 276, 25742–25752
11. Duplus, E., Glorian, M., and Forest, C. (2000) J. Biol. Chem. 275, 15736–15744
12. Alland, L., Muhle, R., Hou, H., Jr., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) Nature 387, 49–55
13. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) Nature 387, 43–48
14. Nagy, L., Kao, H. Y., Chakravarti, D., Liu, R. J., Haisig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) Cell 89, 373–380
15. Hood, Z., Li, R., Barth, P., and Nelson, B. D. (2000) Biochem. J. 346, 93–97
16. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Yoshino, T., Okuyama, Y., Fujita, T., Ohtani-Fujita, N., Matsukawa, Y., Tokino, T., Yamagishi, H., Oka, T., Nomura, H., and Sakai, T. (1997) J. Biol. Chem. 272, 22199–22206
17. Washner, C., Grempler, R., Walther, R., and Schmoll, D. (2001) Biochem. Physiol. Acta 1521, 126–129
18. Jiang, G., Nepomuceno, L., Hopkins, K., and Sladek, F. M. (1995) Mol. Cell. Biol. 15, 5133–5143
19. Zhang, W., Mirkovitch, J., and Darnell, J. E., Jr. (1994) Mol. Cell. Biol. 14, 7276–7284
20. Costa, R. H., Grayson, D. R., and Darnell, J. E., Jr. (1989) Mol. Cell. Biol. 9, 1415–1425
21. Xanthopoulos, K. G., Prezioso, V. R., Chen, W. S., Sladek, F. M., Cortese, R., and Darnell, J. E., Jr. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3807–3811
22. Tian, J. M., and Schibler, U. (1991) Genes Dev. 5, 2225–2234
23. Sath, G. F., and Weiss, M. C. (1997) Mol. Cell. Biol. 17, 1913–1922
24. Liu, Z. and Towle, H. C. (1995) Biochem. J. 308, 105–111
25. Ruse, M. D., Jr., Privalsky, M. L., and Sladek, F. M. (2002) Mol. Cell. Biol. 22, 1626–1638
26. Soutoglou, E., Katrakili, N., and Talianidis, I. (2000) Mol. Cell 5, 745–751

Hepatic Glucose-6-phosphatase Gene Expression 40701