Malic enzyme gene in chick embryo hepatocytes in culture: clofibrate regulates responsiveness to triiodothyronine

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Abstract In chick embryo hepatocytes, triiodothyronine (T3) causes a 30- to 40-fold increase in malic enzyme activity when added between 1 and 3 days, but has no effect when added between 5 and 7 days in culture. This transcription-mediated decline in T3 responsiveness is partially reversed by corticosterone (Roncero, C. and A. G. Goodridge. 1992. *Arch. Biochem. Biophys.* **295:** 258–267). Clofibrate also reversed the decline in responsiveness to T3, and did so in the absence of an increase in binding of T3 to nuclear receptors. The effects of clofibrate and corticosterone were additive, suggesting different mechanisms. The responsiveness of a gene to a specific agent depends on specific regulatory sequences of DNA in that gene. When 5.8 kb of the 5′-flanking DNA of the malic enzyme gene was linked to the chloramphenicol acetyltransferase (CAT) gene and transfected into hepatocytes, T3 stimulated CAT activity. Responsiveness of CAT activity to T3 decreased with time, and this decrease was partially reversed by clofibrate. The T3 responses of cells transfected with various chimeric DNAs that contained T3 response elements (T3REs) of the malic enzyme gene or synthetic consensus T3REs also were increased by clofibrate. The results suggest that clofibrate regulates expression of a metabolite or a protein factor which, in turn, influences function of the T3 receptor.—**Carlisle, T. L., C. Roncero, C. El Khadir-Mounier, D. C. Thurmond, and A. G. Goodridge.** Malic enzyme gene in chick embryo hepatocytes in culture: clofibrate regulates responsiveness to triiodothyronine. *J. Lipid Res.* 1996. **37:** 2088–2097.

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Malic enzyme [EC 1.1.1.40] catalyzes the oxidative decarboxylation of malate to pyruvate and CO_2 simultaneously generating NADPH from $NADP^+$. In avian liver, most of the NADPH used by fatty acid synthase to catalyze the synthesis of palmitate is generated by malic enzyme (1). Malic enzyme activity is low in starved animals and high in fed ones, especially when the diet is high in carbohydrate (1). The stimulation of malic enzyme activity caused by feeding can be mimicked quantitatively in chick embryo hepatocytes in culture by adding triiodothyronine (T3) to the medium or by removing glucagon or unesterified fatty acids from a medium that contains T3 (2–4). The effects of nutritional state in vivo and of T3, glucagon, and fatty acids in hepatocytes in culture are mediated at the level of transcription (4–7).

Between 20 and 68 h in culture, addition of T3 to chick-embryo hepatocytes causes a 30- to 40-fold increase in abundance of malic enzyme mRNA; T3 between 116 and 164 h of incubation has no effect (8). The decrease in T3 responsiveness has a half-life of about 20 h, consistent with a decline in the concentration of a stimulatory protein, the synthesis of which stopped when the hepatocytes were removed from the embryo and placed in culture (or increase in the concentration of an inhibitory protein not present in vivo and induced by culture conditions).

In the absence of T3, corticosterone from the beginning of the incubation has no effect or a small stimulatory effect on the abundance of malic enzyme mRNA; it also has no effect on the accumulation of malic enzyme mRNA caused by adding T3 from 1 to 3 days in culture. However, corticosterone substantially reverses the decrease in T3 sensitivity that occurs with time of incubation (8). Neither the decline in responsiveness to T3 nor the increase in responsiveness caused by corticosterone is associated with changes in binding of T3 to nuclear receptors, suggesting that the T3 receptor itself may not be the putative protein that is declining in concentration (8).

Abbreviations: CAT, chloramphenicol acetyltransferase; PPAR, peroxisomal proliferator-activated receptor; RXR, retinoid X receptor; T3, triiodothyronine; T3RE, T3 response element; TK, thymidine kinase.

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The hypolipidemic drug, clofibrate, is one of several structurally diverse chemical agents that cause increased proliferation of peroxisomes in the liver. Clofibrate and other peroxisomal proliferating agents stimulate accumulation of a number of hepatic proteins, including several peroxisomal and mitochondrial enzymes involved in lipid metabolism (9, 10). When rats are fed with peroxisomal proliferators or rat hepatocytes are treated with such drugs (11–14), malic enzyme activity is increased several-fold. Dehydroepiandrosterone, a peroxisomal proliferator structurally unrelated to clofibrate, stimulates transcription of the malic enzyme gene in rat liver (15). Peroxisomal proliferating agents also cause an increase in abundance of malic enzyme mRNA in rat hepatocytes in culture (13). In this study, we examine the mechanisms by which clofibrate regulates expression of malic enzyme in chick embryo hepatocytes.

MATERIALS AND METHODS

Materials

Clofibric acid (2-(*p*-chlorophenoxy)-2-methylpropionic acid), fenofibrate (2-[4-(4-chlorobenzoyl)phenoxy]-2-methylpropanoic acid), bezafibrate (2-[4-(2-[4-chlorobenzamido]ethyl)-phenoxy]-2-methylpropanoic acid), and 3,5,3′ L-triiodothyronine were purchased from Sigma Chemical Co. Methylclofenapate (methyl-2-[4-(*p*-chlorophenyl)phenoxy]-2-methylpropionate) was obtained from Lancaster Synthesis Ltd., Morecambe, England. Crystalline bovine insulin (Eli Lilly and Co.; Indianapolis, IN), collagenase (Boehringer Mannheim Biochemicals), SP6-grade [^{32}P]-UTP (800 Ci/mmol) (Amersham Corp.) and [^{32}P]-dCTP (800 Ci/mmol) (ICN Biochemicals), and nucleotides used in the transcription assays (Pharmacia) were obtained from the indicated sources. *E. coli* cells of strain DH5α were used for subcloning and large-scale preparations of plasmid DNA. LipofectAce™ and Waymouth medium MD 705/1 were purchased from Gibco/BRL. All other chemicals were of reagent grade or of the highest purity commercially available.

Preparation and maintenance of isolated cells

Unincubated embryonated eggs from white Leghorn chickens were obtained from HyVac Laboratory Eggs, Gowrie, IA, and incubated in an electric forced-draft incubator at 37.5 ± 0.5°C. Hepatocytes were prepared from the chopped livers of 19-day-old chick embryos by incubation with collagenase, isolated by centrifugation, and incubated at 40°C under an atmosphere of 5% CO_2 in air (8). The cells were incubated in plastic tissue-culture dishes of 35- or 60-mm diameter in 2 or 5 ml, respectively, with Waymouth medium MD 705/1 supplemented with penicillin G (60 μg/ml), streptomycin (100 μg/ml), and insulin (50 nM). At about 20 h of incubation, the medium was changed to one of the same composition, with or without addition of clofibrate or other compounds or hormones as indicated in the legends to the figures and tables. Unless otherwise noted, the medium was changed every 48 h thereafter.

Northern blot analysis

Total RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method (16) and analyzed as previously described (8), using a modification of the method of Amasino (17) for prehybridization and hybridization. RNA was extracted from the pooled cells of three 35-mm plates, separated by size on gels containing 0.9% agarose and 37% formaldehyde, and then transferred to GeneScreen™ membranes (NEN Research Products) using a VacuGene blotting apparatus (LKB Instruments). RNA was cross-linked to the membrane by UV light. Probe DNAs were labeled with [^{32}P]dCTP by nick translation (Bethesda Research Laboratories [18]). The resulting membranes were subjected to autoradiography for 1–3 days. Different exposure times were used for densitometric quantitation of the hybridization signals at 633 nm in a LKB UltraScan XL densitometer.

Nuclear run-on transcription assays

Nuclear run-on transcription assays were performed as described previously (8). Briefly, nuclei were isolated from the pooled cells of 20–24 60-mm plates (6, 19). Approximately 4 × 10^7 nuclei were stored at -70°C in 100-μl portions in 50 mM HEPES, pH 7.9, 75 mM NaCl, 0.1 mM ETDA, 5 mM dithiothreitol, 0.125 mM phenylmethylsulfonyl fluoride, and 50% glycerol. The in vitro elongation reactions were carried out as described (7, 20). ^{32}P-labeled RNA transcripts were purified (21) using NICK columns (Pharmacia). DNA probes were denatured and applied to GeneScreen™ membranes (2 μg/slot) using a slot-blot apparatus (Bethesda Research Laboratories). ^{32}P-labeled RNA (2–3 × 10^7 cpm/ml) was hybridized to DNA on slot-blotted membranes for 2 days at 65°C in 10 mM HEPES, pH 7.5, 10 mM EDTA, 0.3 M NaCl, 1% SDS, 0.02% polyvinylpyrrolidone (M_r 40,000), 0.02% ficoll (M_r 40,000), 0.02% bovine serum albumin (fraction V), and 250 μg/ml yeast tRNA. The membranes were washed and incubated with RNase A, and then washed again and subjected to autoradiography.

DNA probes

The chicken cDNAs used in these experiments were malic enzyme (22), β-actin (23), glyceraldehyde-3-phosphate dehydrogenase (24), T3 receptor α (25), and retinoid X receptor γ (RXR γ) (26). Chicken malic enzyme DNAs used in the nuclear run-on assays are subclones of genomic DNAs cloned in phage-λ vectors (27). ME-4.8–5′ (4.8 kb in pUC19) is an *Eco*RI fragment from λ-clone 20B and is derived from the most 5′ intron of the malic enzyme gene. ME-4.8–3′ (4.8 kb in pUC19) is an *Eco*RI fragment of λ-clone 2B and contains exon 8 plus surrounding intronic DNA and is from the middle of the mRNA. ME-2.6 (2.6 kb in M13mp18) is an *Eco*RI fragment of λ-clone 1 and contains intron DNA and exons 11 and 12 from the 3′ one-third of the mRNA.

Transient transfection

p[ME-5800/+31]CAT, p[ME-4135/-2715]TKCAT, p[ME-3903/-3617]TKCAT, and p[ME-3883/-3858]TKCAT were constructed as described (27). pTRE[DR4]$_5$TKCAT contains 5 repeats of the synthetic oligomer, 5′GGAGCTTCAGGTCACAGGAGGTCAGAGAGCT3′. Half-sites of this direct repeat with 4-bp spacing are underlined. Transfections were carried out by a modification of published procedures (28). Briefly, 2.5 μg p[ME-5800/+31]CAT or an equimolar amount of another reporter plasmid, 0.5 μg pCMV β-GAL (29), and pBluescript (to bring total DNA to 3.0 μg per plate) were transfected. Cells were prepared as described above and plated on day zero. At about 20 or 68 h of incubation, the medium was replaced with one containing a mixture of DNAs and LipofectAce™. After 6 h of incubation, the transfection medium was replaced with fresh medium with or without T3 (1.6 μM). After 46 h, hepatocytes were harvested and cell extracts were prepared.

Analysis of cell extracts

For the assay of malic enzyme activity and amounts of protein and DNA, cell extracts were prepared by homogenizing cells in ice-cold 0.25 M sucrose, 0.010 M Tris HCl, pH 7.4, 1 mM dithiothreitol with 20 strokes of a Dounce homogenizer with a tight-fitting (B) pestle, followed by centrifugation (27,000 $g \times 30$ min). Total extracts and cell fractions were stored at -80°C. Malic enzyme activity (30), DNA (31), and protein (32) were assayed by the indicated methods. Isocitrate dehydrogenase activity was measured by substituting 5 mM isocitrate for malate in the assay for malic enzyme activity.

For measurements of CAT (33) and β-galactosidase (34) activities, cell extracts were prepared by three cycles of freezing and thawing, followed by centrifugation to remove particulate material (28). For CAT activity, extracts were heated to 60°C for 30 min; precipitated protein was removed by centrifugation. Samples of heat-stable extract containing the equivalent of 2 to 50 μg of unheated soluble protein were incubated for 15 h at 37°C in 2.6 mM acetyl-CoA, 100 mM Tris-HCl, 1 mM EDTA, and 12 μM [^{14}C]chloramphenicol. Chloramphenicol and its acetylated products were extracted with ethyl acetate and separated by thin-layer chromatography. Radioactivity in substrate and products was measured by liquid scintillation spectrometry. The results were expressed initially as percent of substrate converted to acetylated product per microgram unheated soluble protein and then normalized to β-galactosidase activity (34) in the same extracts.

Western blot analysis

Chick embryo hepatocytes were incubated with insulin (50 nM) alone or insulin plus corticosterone (1 μM) or insulin plus clofibrate (1 mM). At about 20 h of incubation, the medium was changed to one of the same composition with or without T3 (1.6 μM). After 48 h of incubation, the cells were harvested, and nuclear extracts were prepared (35). Nuclear proteins (40 μg) were separated by size by electrophoresis on 10% SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose membranes (Millipore Corporation, Bedford, MA). The immunoblot was performed according to the procedure provided by the manufacturer of the kit (ECL Western blotting protocol, Amersham Life Science, Arlington, IL). The primary antibodies were polyclonal, anti-chicken TRα1 and anti-human retinoid X receptor β (RXRβ) (both from Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The second antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Life Science, Arlington, IL). Both preparations were diluted 1:5000.

Statistical analysis

Where appropriate, statistical significance between means was determined by the non-parametric Wilcoxon matched-pairs, signed-rank test (36). Standard errors of the mean are provided to indicate the degree of variability in the data.

RESULTS

Clofibrate regulates responsiveness to T3

Clofibrate had a small (but statistically significant) positive effect on the activity of malic enzyme in hepatocytes incubated in Waymouth medium containing insulin for 68 or 116 h (**Table 1**). Clofibrate caused 1.4-

TABLE 1. Effect of corticosterone and clofibrate on malic enzyme activity in chick embryo hepatocytes in culture

	T3 (20 to 68 h)				T3 (68 to 116 h)			
Treatment	Ins	Ins + T3	Ins + Cort	Ins + T3 + Cort	Ins	Ins + T3	Ins + Cort	Ins + T3 + Cort
None	0.11 ± 0.01	3.5 ± 0.3[a]	0.08 ± 0.01[a]	4.6 ± 0.4[a,b,c]	0.15 ± 0.01[a]	0.34 ± 0.06[b,d]	0.13 ± 0.01[e]	3.8 ± 0.4[d,f,g]
Clofibrate	0.15 ± 0.02[a]	4.9 ± 0.6[h,i]	0.17 ± 0.02[c]	6.2 ± 0.5[h,j,k,l]	0.21 ± 0.03[m,n]	2.8 ± 0.6[i,o]	0.21 ± 0.02[g]	6.0 ± 0.6[o,p,q]

Hepatocytes were isolated from the livers of 19-day-old chick embryos as described in Materials and Methods and incubated in a chemically defined medium containing insulin (50 nM). After 20 h of incubation, the medium was changed to one of the same composition or one of the same composition plus corticosterone (1 μM) or clofibrate (1 mM); plates of cells of each group were treated with T3 (1.6 μM). Cells from T3-treated plates and a corresponding number of control plates from each group were harvested at 68 h of incubation. At 68 h of incubation, additional sets of plates containing control, corticosterone-treated, or clofibrate-treated hepatocytes were treated with T3 or no additional hormones. Cells from these plates were harvested at 116 h of incubation. Malic enzyme activity and DNA were measured as described in Materials and Methods. The results are expressed as units of malic enzyme activity per milligram of DNA and represent the mean ± SEM of 9 independent experiments (each using a different preparation of hepatocytes); each experiment was performed in duplicate. The superscript letters indicate statistical significance of differences as follows: *a*, $P < 0.01$ vs. Ins 68 h; *b*, $P < 0.01$ vs. Ins + T3 68 h; *c*, $P < 0.01$ vs. Ins + cort 68 h; *d*, $P < 0.01$ vs. Ins 116 h; *e*, $P = 0.02$ vs. Ins 116 h; *f*, $P < 0.01$ vs. Ins + T3 116 h; *g*, $P < 0.01$ vs. Ins + cort 116 h; *h*, $P < 0.01$ vs. Ins + clofibrate 68 h; *i*, $P < 0.05$ vs. Ins + T3 68 h; *j*, $P < 0.01$ vs. Ins + T3 + clofibrate 68 h; *k*, $P < 0.01$ vs. Ins + cort + clofibrate 68 h; *l*, $P < 0.05$ vs. Ins + cort + T3 68 h; *m*, $P < 0.01$ vs. Ins 116; *n*, $P = 0.02$ vs. Ins + clofibrate 68 h; *o*, $P < 0.01$ vs. Ins + clofibrate 116 h; *p*, $P < 0.01$ vs. Ins + cort + clofibrate 116 h; *q*, $P < 0.01$ vs. Ins + T3 + cort 116 h. Ins, insulin; Cort, corticosterone; T3, triiodothyronine.

and 8-fold increases in the responses of hepatocytes to T3 added from 20 to 68 h and 68 to 116 h, respectively. As demonstrated previously (8), corticosterone had little effect on T3 responsiveness between 20 and 68 h and a 10-fold effect between 68 and 116 h (Table 1). When corticosterone and clofibrate were added together at near maximally effective doses, their effects on T3-induced malic enzyme activity were additive, suggesting that these agents act by independent mechanisms. Clofibrate had no effects on DNA or protein per plate or isocitrate dehydrogenase activity after 3 or 5 days of incubation (results not shown).

The effects of clofibrate on T3-induced malic enzyme activity were dose-dependent (**Fig. 1**). As little as 60 μM clofibrate caused about a 2-fold effect. Assuming maximum responsiveness at about 2 mM, the EC_{50} for clofibrate was between 0.5 and 1 mM. The response of malic enzyme to clofibrate likely represents a response to the class of drugs known as peroxisomal proliferators, because methylclofenopate, nafenopin, bezafibrate, and fenofibrate, other members of that class of drugs also increased responsiveness of chick embryo hepatocytes to T3 added between 68 and 116 h (**Table 2**).

Clofibrate regulates T3 responsiveness at the level of transcription

The decrease in responsiveness of malic enzyme activity to T3 that occurs during the first 5 days of culture and its partial reversal by corticosterone is accompanied by comparable changes in T3 responsiveness of the abundance of malic enzyme mRNA and the rate of transcription of the malic enzyme gene (8). Therefore, we next examined the effect of clofibrate on the abundances of the mRNAs for malic enzyme (**Fig. 2**). Neither T3 nor clofibrate affected the abundance of glyceraldehyde-3-phosphate dehydrogenase. This constitutively expressed gene was thus a good control for selectivity of the response to T3 and clofibrate.

The pattern of responsiveness of malic enzyme mRNA was similar to that for malic enzyme activity. When T3 was added between 20 and 68 h, there was a large increase in the abundance of malic enzyme mRNA. This effect of T3 was larger when clofibrate was also

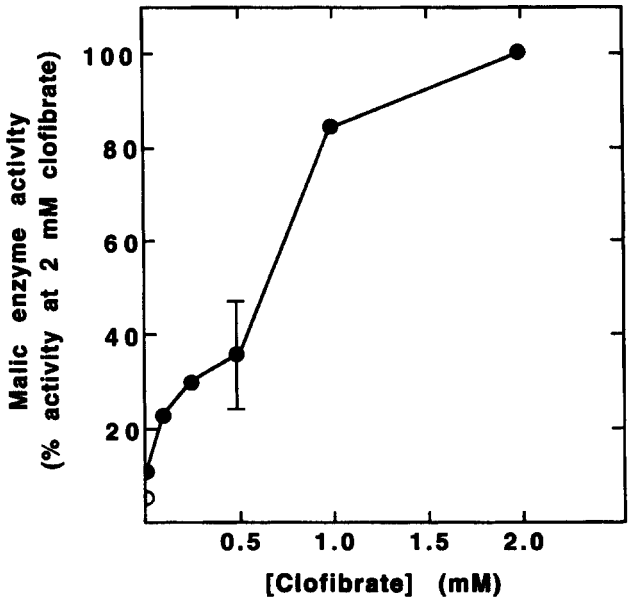

Fig. 1. Responsiveness of malic enzyme activity to T3 as a function of clofibrate concentration. Hepatocytes were isolated as described in the legend to Table 1 and incubated in culture for 20 h in the presence of insulin (50 nM). At 20 h incubation, the media were changed to media of the same composition plus clofibrate at the indicated concentrations. At 68 h of incubation, T3 (1.5 μM, closed circles) was added to half of the plates. At 116 h of incubation, the cells were harvested and assayed for malic enzyme activity and DNA. The results were originally expressed as units of malic enzyme activity per milligram of DNA and then were normalized to the values for 2.0 mM clofibrate (100%). The results represent the averages of two different experiments. Open half circle, no T3.

TABLE 2. Effect of various fibrate derivatives on responsiveness of malic enzyme activity to T3

Treatment	T3 (68 to 116 h)			
	Ins	Ins + T3,	Ratio	n
None	10.6 ± 1.6	43 ± 7	5.3 ± 1.5	11
Clofibrate (1 mM)	8.3 ± 1.2	137 ± 29	18 ± 6	4
Methylclofenopate	7.8 ± 1.6	199 ± 56	32 ± 13	4
Nafenopin	12 ± 1	145 ± 22	12 ± 4	3
Bezafibrate	14 ± 3	129 ± 44	9.1 ± 1.3	2
Fenofibrate	19 ± 7	287 ± 98	15 ± 0.1	2

Isolation and incubation of hepatocytes and expression of the results were described in the legend to Table 1, except that malic enzyme activity is expressed as milliunits per milligram soluble protein. Only results from 68 to 116 h are presented. Clofibrate, 1 mM; nafenopin, 500 μM; methylclofenapate, bezafibrate, and fenofibrate, 100 μM.

present. Between 68 and 116 h, T3 caused a barely detectable increase in the abundance of malic enzyme mRNA. When clofibrate was present, however, the response of T3 was almost as great as that in hepatocytes incubated with T3 and clofibrate from 20 to 68 h. Clofibrate had no detectable effect on malic enzyme mRNA in the absence of T3.

The transcription measurements were made only in cells incubated with T3 from 68 to 116 h because we had already established that the time-dependent decrease in malic enzyme activity was correlated with a similar decrease in rate of transcription of the malic enzyme gene (8). Transcription of the malic enzyme gene was measured using probes from the 5′, middle, and 3′ ends of this large (> 108 kb [27]) gene. We also measured transcription of two control genes, glyceraldehyde-3-phosphate dehydrogenase and β-actin (**Fig. 3; Table 3**). In general, the pattern of response for transcription of the malic enzyme gene was the same for each of the malic enzyme probes and the same as those for malic enzyme activity and abundance of malic enzyme mRNA. In the absence of clofibrate, addition of T3 from 68 to 116 h of incubation caused a 30% increase in transcription of the malic enzyme gene (average of rates with the three probes). Unexpectedly, clofibrate caused an 81–84% decrease in transcription of the malic enzyme gene in the absence of T3. This effect was not detected with malic enzyme activity or mRNA abundance; this difference may reflect the difficulty of measuring a decrease in activity or mRNA level when values are already near background. In T3-treated hepatocytes, clofibrate caused about a 7-fold increase in transcription of the malic enzyme gene. Because basal activity was decreased, the T3 effect in clofibrate-treated cells was more than 40-fold (Fig. 3, Table 3).

Neither the decrease in responsiveness of the malic enzyme gene to T3 nor the partial reversal of that responsiveness by corticosterone were accompanied by changes in T3 binding (4). Similarly, clofibrate for 24 or 48 h did not significantly affect binding of T3 its nuclear receptors (**Table 4**).

Localization of DNA response elements that confer responsiveness to clofibrate

Clofibrate could influence function of the T3 receptor by controlling the intracellular concentration of a regulatory metabolite or by controlling the concentration or activity of a regulatory protein. Peroxisomal proliferators influence the levels of numerous proteins and enzymes and, consequently, the concentrations of the substrates and products of the affected metabolic pathways (9, 10). Thus, identification of the relevant regulatory factor(s) will be exceptionally difficult in the complex environment of intact hepatocytes. As a consequence, we decided to take another approach to understanding the molecular events by which clofibrate exerts its effects on T3 responsiveness of the transcription of the malic enzyme gene. Whether or not a gene responds

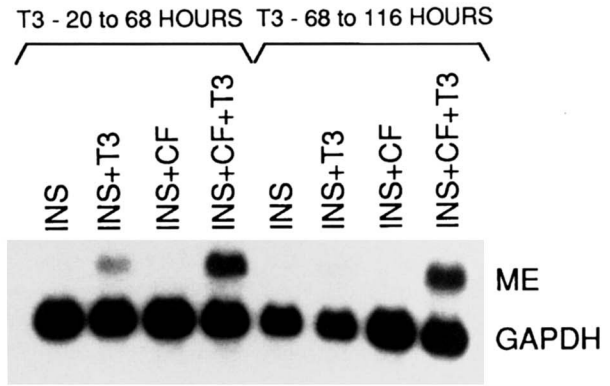

Fig. 2. Abundances of the mRNAs for malic enzyme and glyceraldehyde-3-phosphate dehydrogenase in cells incubated with T3 for 48 h and with or without clofibrate from 20 h of incubation. Hepatocytes were isolated, incubated in culture, treated with hormones, and harvested as described in the legend to Table 1 (INS, insulin, CF, clofibrate).

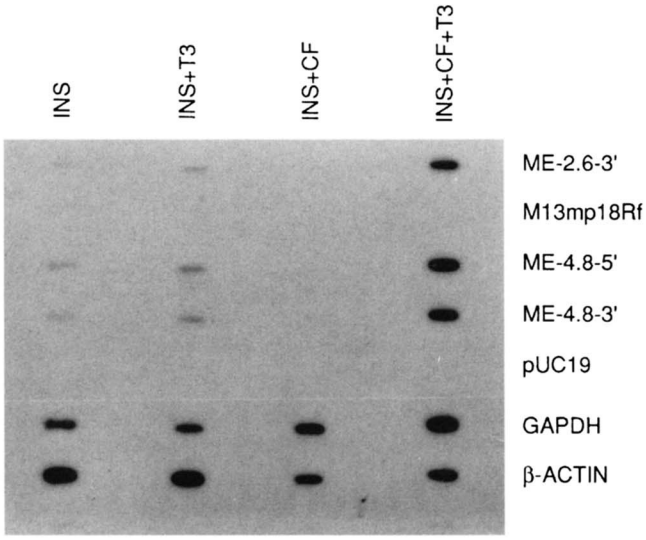

Fig. 3. Transcription of the genes for malic enzyme, β-actin, and glyceraldehyde-3-phosphate dehydrogenase in cells treated with T3 for 48 h, with and without clofibrate from 20 h of incubation. Other details are given in the legend to Table 3.

to a specific hormone or drug depends on the presence or absence of specific sequences of DNA in the regulatory regions of that gene, usually in the 5′-flanking DNA. When 5.8 kb of 5′-flanking DNA of the malic enzyme gene is linked to the reporter gene, chloramphenicol acetyltransferase (CAT), and introduced into chick embryo hepatocytes by transfection, T3 causes a large increase in CAT activity (27, 37). Through a series of deletion and substitution mutations, we have identified a 135-bp region (-3903 to -3769 bp) that we call a T3 response unit; it contains one major (-3883 to -3858 bp) and two or three weak T3 response elements (T3REs) (27). These response elements confer T3 responsiveness on both the homologous promoter of the malic enzyme gene and the heterologous promoter of the thymidine kinase (TK) gene of herpes simplex virus. We have used some of the DNA constructs that we used to characterize the malic enzyme T3REs to localize sequences that confer responsiveness to clofibrate.

Stimulation of CAT activity by T3 in hepatocytes transfected with constructs containing 5.8 kb of 5′-flanking DNA of the malic enzyme gene decreased as a function of time in culture (**Table 5**). When the cells were treated with T3 from 26 to 72 h, T3 caused a 5-fold increase in CAT activity in the absence of clofibrate and a 10-fold increase in its presence. T3 treatment between 74 and 120 h of incubation did not have a statistically significant effect on CAT activity in the absence of clofibrate, but caused a 5-fold increase in CAT activity in hepatocytes incubated with clofibrate. This result indicates that the DNA sequence elements responsible for the decline in T3 responsiveness as a function of time in culture and for the effects of clofibrate on T3 responsiveness are present in the 5.8 kb upstream of the start site for transcription of the malic enzyme gene.

In the next three sets of transfections, we used constructs that had fragments of the malic enzyme gene linked to TK. In each case, responsiveness to T3 was much weaker between 74 and 120 h than between 26 and 72 h (Table 5). In hepatocytes transfected with each of these constructs, the decline in responsiveness to T3 was much smaller when clofibrate was present. Preservation of the T3 response was similar in magnitude for cells transfected with constructs driving malic enzyme or TK promoters. In hepatocytes transfected with pME[-4135/-2715]TKCAT, clofibrate caused a 3-fold increase in malic enzyme activity in cells incubated with T3 from 74 to 120 h (results not shown); the effect of clofibrate on CAT activity in T3-treated cells was 2-fold. Thus, all sequence elements required for the action of clofibrate appear to reside in this fragment of DNA. Construct pME[-4135/-2715]TKCAT contains the T3REs described above plus a second weaker T3RE closer to its 3′ end.

The presence of T3REs was common to all of the constructs that conferred T3 responsiveness on CAT activity in transfected hepatocytes. Plasmid ME[-3903/-3617]TKCAT contains the T3 response unit described above plus some additional 3′ sequence. Plasmid ME[-3883/-3858]TKCAT contains only the strong T3RE plus

TABLE 3. Transcription of the gene for malic enzyme in cells treated with T3 from 68 to 116 h of incubation, with or without clofibrate from 20 h of incubation

Gene Fragment	Ins	Ins + T3	Ins + Clofibrate	Ins + T3 + Clofibrate
Malic enzyme - 4.8 kb-5′	68 ± 21	100	13 ± 7	570 ± 350
Malic enzyme - 4.8	80 ± 34	100	13 ± 7	760 ± 320
Malic enzyme - 2.6 kb-3′	85 ± 31	100	16 ± 8	690 ± 480

Hepatocytes were isolated, incubated, treated with hormones, and harvested as described in the legend to Table 1 (Ins, insulin, T3, triiodothyronine). Nuclei were isolated and transcription run-on assays were carried out as described under Materials and Methods. Results were originally expressed as arbitrary units per reaction. They were then corrected for differences in total transcription by normalizing to transcription of the β-actin gene (arbitrary units hybridizing to a malic enzyme DNA fragment divided by arbitrary units hybridizing to β-actin cDNA). Finally, all of the results were expressed relative to that for cells treated with insulin and T3 (100%). The results are the averages ± SEM of three experiments. Similar results were obtained when the results were normalized to transcription of the glyceraldehyde-3-phosphate dehydrogenase gene

TABLE 4. Effect of clofibrate (1 mM) on T3 binding to nuclear receptors in hepatocytes in culture

Hours with Clofibrate	Insulin	Insulin + Clofibrate
24	170 ± 4 (6)	130 ± 10 (6)
48	160 ± 3 (6)	100 ± 10 (3)

The T3 concentration was 200 pM. Results are expressed as fmols ^{125}I-labeled T3 bound per mg DNA ± SEM of the number of experiments indicated in parentheses.

5 bp of flanking DNA upstream and downstream of that T3RE (27). We also prepared a construct containing five copies of a consensus T3RE (5′-AGGTCANNNNAG-GTCA-3′) linked to TKCAT. This construct did not contain any sequence from the malic enzyme gene. In hepatocytes transfected with this construct, T3 caused a 40-fold increase in CAT activity when the hormone was present between 26 and 72 h. Between 74 and 120 hours, the increase was only 4.5-fold. In the presence of clofibrate, T3 responsiveness in the later period was increased to 10-fold (Table 5). Thus, every construct that contained a functional T3RE also contained the response elements required for loss of responsiveness with time in culture and for the increase in responsiveness caused by clofibrate; malic enzyme DNA per se was not required.

DISCUSSION

One explanation of our results is that the activity or concentration of a positive transcription factor involved in the T3 response is lost after the cells are placed in culture (accumulation of a negative factor is equally

TABLE 5. Effects of clofibrate and T3 on CAT activity in hepatocytes transfected with various expression plasmids

	T3 (26 to 72 h)					
	No Clofibrate			Clofibrate (1 mM)		
Construct	Ins	Ins +T3	Fold-Effect	Ins	Ins +T3	Fold-Effect
pME-5800/+31CAT	1.6 ± 0.6	6.0 ± 1.7	5.3 ± 1.2	1.0 ± 0.3	8.5 ± 1.6	9.6 ± 1.2[a]
pME[-4135/-2715] TKCAT	1.6 ± 0.5	46 ± 13	49 ± 8	1.0 ± 0.2[a]	93 ± 32[a]	94 ± 17[a]
pME[-3903/-3617] TKCAT	0.73 ± 0.24	30 ± 7	46 ± 11	0.53 ± 0.11	30 ± 7	47 ± 8
pME[-3883/-3858] TKCAT	0.44 ± 0.11	17 ± 3	66 ± 12	0.41 ± 0.07	24 ± 6	80 ± 25
pTRE[DR4]$_5$ TKCAT	0.26 ± 0.11	4.2 ± 0.6	40 ± 11	0.70 ± 0.51	7.6 ± 1.8	52 ± 13
	T3 (74-120 h)					
	No Clofibrate			Clofibrate (1 mM)		
Construct	Ins	Ins +T3	Fold-Effect	Ins	Ins +T3	Fold-Effect
pME-5800/+31CAT	3.1 ± 1.4	3.1 ± 1.1	1.4 ± 0.2	2.5 ± 1.3[b]	7.4 ± 2.5[a]	5.4 ± 1.1[a]
pME[-4135/-2715] TKCAT	1.3 ± 0.3	14 ± 5	11 ± 2	1.6 ± 0.5	27 ± 7[a]	25 ± 3[a]
pME[-3903/-3617] TKCAT	2.1 ± 0.9	16 ± 7	9 ± 3	1.9 ± 1.0	28 ± 11	26 ± 6[a]
pME[-3883/-3858] TKCAT	3.7 ± 1.0	16 ± 3	5 ± 0.6	4.5 ± 0.9	69 ± 16[a]	16 ± 2[a]
pTRE[DR4]$_5$ TKCAT	1.1 ± 0.4	2.2 ± 0.6	4.5 ± 1.0	0.4 ± 0.1[a]	3.0 ± 0.6	10 ± 2[a]

Isolation of hepatocytes and incubation in culture were carried out as described in Materials and Methods. Where present, clofibrate was added at 20 h of incubation at 1 mM. The number at the left of each construct indicates the 5′ end of malic enzyme DNA in nucleotides relative to the major start site for transcription; for all malic enzyme constructs the 3′ end of malic enzyme DNA was at +31. Chick embryo hepatocytes were transiently transfected using LipofectACE™ (30–40 μg/plate) and p[ME-5800/+31]CAT (2.5 μg/plate or an equimolar amount of the other constructs), pCMVβ-GAL (0.5 μg/plate) and pBluescript or pKSCAT (promoterless construct) DNA (sufficient to bring total DNA per plate to 3.0 μg) as described in Materials and Methods. The results were expressed as percentage of [^{14}C]chloramphenicol converted to acetylated chloramphenicol per microgram soluble protein and then corrected for differences in transfection efficiency by dividing by β-galactosidase activity of the same extract (A_{420} units per μg protein). T3 had no effect on β-galactosidase activity. For each construct, fold response to T3 was calculated by dividing the corrected CAT activity for hepatocytes treated with T3 by that for hepatocytes not treated with T3. The fold responses were calculated for individual experiments and then averaged; they are not the same as the quotients of the averaged CAT activities. The results are the means ± SEM of 7 to 16 experiments, each one using an independently isolated batch of hepatocytes. [a]$P < 0.02$ versus same day with no clofibrate; [b]$P < 0.05$ versus same day with no clofibrate. TK, minimal promoter of herpes simplex virus thymidine kinase; DR_5, five copies of a consensus T3RE (AGGTCANNNNAGGTCA).

possible) and that either the activity or concentration of that same factor or another with similar function is induced (or inhibited if a negative factor) by clofibrate. Alternatively, the protein that is lost as a function of time in culture and induced by clofibrate may be an enzyme that catalyzes production of a ligand that regulates the activity of a transcription factor. The transcription factor we postulate could bind to DNA independently of the T3 receptor or could bind to and/or regulate a function of the T3 receptor itself. If the factor were to bind independently, then both a T3RE and another sequence would be required for the action of clofibrate on T3 responsiveness. p[ME-3883/-3858]TKCAT contains a T3RE and little additional sequence; pTRE[DR4]$_5$TKCAT does not contain any malic enzyme sequence. Thus, only a T3RE was necessary. These results strongly suggest that the decrease in responsiveness to T3 that occurs as a function of time in culture and the reversal of that decrease caused by clofibrate involve factors that interact directly with T3REs.

T3REs in the direct-repeat orientation selectively recognize homodimers of the T3 receptor and heterodimers of T3 receptor with RXR (38) or RXR-related factors (39) in liver. T3 receptor–RXR heterodimers bind more tightly to DNA and are more effective transcriptional activators than homodimers of T3 receptor (40). Loss of T3 receptor itself or of RXR or a related factor from the cells in culture and stimulation of its accumulation by clofibrate or corticosterone, therefore, could explain the changes in T3 responsiveness that we have observed in hepatocytes in culture. The lack of any change in T3 binding during the period when T3 responsiveness is declining (8) is inconsistent with changes in the concentration of the T3 receptor itself being involved. Furthermore, clofibrate had no effect on the nuclear levels of the T3 receptor or RXR (western blot analyses) or the abundances of the mRNAs for the T3 receptor or RXR (northern blot analyses) (results not shown). Are there other factors that could regulate responsiveness to T3?

The peroxisomal proliferator activated receptor (PPAR) was cloned by virtue of its sequence similarity to members of the steroid/thyroid receptor gene family and the ability of its ligand binding domain to confer transcriptional responsiveness to peroxisomal proliferating agents (41). Subsequently, PPARs have been discovered in a number of different organisms (42–44). Transcription of the PPAR gene in rat liver is stimulated by glucocorticoids (45) and fenofibrate, a peroxisomal proliferating agent (46). We have not been able to assess the effects of clofibrate on PPAR levels in chick embryo hepatocytes because the chicken cDNA has not been cloned, and we have not been able to measure the level of chicken PPAR with a mammalian PPAR cDNA. Nevertheless, two reports suggest that the activity or concentration of PPAR is unlikely to be involved in clofibrate-mediated regulation of T3 responsiveness. First, PPAR and T3 receptor can heterodimerize, but the heterodimers do not bind to direct-repeat T3REs with 4-bp spacing. As a consequence, on T3REs with 4-bp spacing, PPAR is a dominant negative regulator and inhibits the action of T3 (47). A positive action of T3 is observed if the T3RE half-site has 2-bp spacing, but this is not the structure of the T3REs of the chicken malic enzyme gene (27) or the artificial T3RE that we tested. Second, PPAR also can form heterodimers with RXR. Because formation of T3 receptor–RXR heterodimers augments the action of T3, increased formation of PPAR–RXR heterodimers should inhibit the action of T3 by lowering the level of T3 receptor–RXR heterodimers (48). Thus, a clofibrate- or corticosterone-induced increase in expression of PPAR seems unlikely to account for their effects on T3 responsiveness of expression of the malic enzyme gene.

In the rat, both in vivo and in isolated hepatocytes, peroxisomal proliferating agents, nafenopin, bezafibrate and Medica 16, stimulate accumulation of malic enzyme mRNA in a manner independent of thyroid hormone (13, 15). For dehydroepiandrosterone, however, the effects on transcription of the malic enzyme gene were dependent on an intact thyroid system; no effect was observed in hypothyroid rats (15). In chick embryo hepatocytes, the peroxisomal proliferating agents that we tested did not cause a biologically meaningful stimulation of expression of malic enzyme independent of added thyroid hormone. The difference in the responsiveness of malic enzyme to peroxisomal proliferating agents between rat and chick embryo hepatocytes could be due to differences in the cell culture conditions. Alternatively, differences in the structures of 5′-flanking DNA of the rat and chicken malic enzyme genes and differences in the mechanisms by which T3 and peroxisomal proliferating agents regulate transcription of the malic enzyme gene may be involved (27). For example, the rat malic enzyme gene probably responds to peroxisomal proliferating agents in the absence of T3 because it contains a PPAR element (49); the chicken gene appears to lack such an element.

The arguments developed in this report suggest that changes in neither the concentration nor the activity of PPAR are likely to mediate the effect of clofibrate on T3 responsiveness of the malic enzyme gene. Our results also suggest that regulation of the concentrations of RXR or the T3 receptor itself do not mediate the clofibrate effect. However, peroxisomal proliferating agents also increase levels of enzymes that control the concentrations of small molecules. These molecules, in turn, could regulate function of the T3 receptor. Such

a small molecule could bind directly to the T3 receptor and regulate its function, or it could interact with a protein that influences transcriptional activity of the T3 receptor at T3REs with 4-bp spacing. Candidates include ligands for RXR or for an as yet undescribed orphan receptor that heterodimerizes with the T3 receptor or a protein that communicates T3-dependent activation from the T3 receptor to the general transcription apparatus.

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