Down-regulation of rat mitochondrial branched-chain 2-oxoacid dehydrogenase kinase gene expression by glucocorticoids

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The mammalian mitochondrial branched-chain 2-oxoacid dehydrogenase (BCOD) complex is regulated by a reversible phosphorylation (inactivation)/dephosphorylation (activation) cycle. In the present study, the effects of glucocorticoids on the level of BCOD kinase mRNA were investigated in rat hepatoma cell lines (H4IIE and FTO-2B), as well as in the rat. In H4IIE cells, dexamethasone was found to significantly reduce steady-state concentrations of BCOD kinase mRNA after a 48 h culture, and this was correlated with a 2-fold increase in the dephosphorylated form of the BCOD complex. The half-life of the kinase mRNA in H4IIE cells was not affected by dexamethasone treatment. Therefore, the decrease in the steady-state kinase mRNA level resulting from dexamethasone treatment was not caused by changes in mRNA stability, which raised the possibility of regulation at the level of gene transcription. To identify the negative glucocorticoid-responsive element in the kinase promoter,

nested deletion constructs in the 3.0 kb promoter region were examined in H4IIE cells cultured in the presence or absence of dexamethasone. No significant differences in promoter activity were observed on either transient or stable transfection. The data showed that the glucocorticoid-responsive element was located outside the 3.0 kb promoter region. At the physiological level, hepatic BCOD kinase mRNA levels were reduced in rats injected intraperitoneally with dexamethasone. This effect was liver-specific, and was not detected in other tissues. These results suggest that the down-regulation of kinase gene expression by glucocorticoids is mediated through a liver-specific or -enriched transcription factor(s).

Key words: dexamethasone, H4IIE cells, kinase mRNA, kinase promoter, liver-specific factor.

INTRODUCTION

The mammalian mitochondrial branched-chain 2-oxoacid dehydrogenase (BCOD) complex catalyses the rate-limiting step of branched-chain amino acid (leucine, isoleucine and valine) catabolism, i.e. the oxidative decarboxylation of branched-chain 2-oxoacids derived from branched-chain amino acids. The BCOD complex comprises multiple copies of three catalytic components: a thiamine pyrophosphate-dependent branched-chain 2-oxoacid decarboxylase (E1) that contains two E1 α and two E1 β subunits; a dihydrolipoyl transacylase (E2); and a dihydrolipoyl dehydrogenase (E3) [1,2]. In addition, the complex has a set of regulatory enzymes: a specific kinase [3] and a specific phosphatase [4], which control enzyme activity through reversible phosphorylation (inactivation)/dephosphorylation (activation) of E1 [5].

The activity of the BCOD complex varies between tissues, and is regulated by nutritional and hormonal stimuli [5–9]. This provides an effective mechanism by which to modulate the flux of branched-chain amino acids through the BCOD complex. For example, administration of glucagon and insulin to rats fed on a low-protein diet resulted in a 3–4-fold increase in the hepatic enzyme activity of the complex, due to an increased percentage of the active dephosphorylated form, i.e. the activity state of the complex [10]. Treatment of rats fed on a low-protein diet with dibutyryl-cAMP also resulted in nearly full activation of the complex. Injection of 6 α -methylprednisolone (a synthetic glucocorticoid) into rats fed on a chow diet resulted in a 5-fold increase in the activity state of the complex in skeletal muscle [11]. However, in these studies, changes in specific activities or amounts of the BCOD kinase and phosphatase that resulted in an increased activity state of the BCOD complex induced by hormones were not determined.

The BCOD kinase activity in the livers of rats fed on protein-free diets has been shown to be increased 3–4-fold compared with that in rats fed on a 50% (w/w) protein diet [7]. The increase in kinase activity was accompanied by corresponding elevations in the levels of the kinase protein and mRNA. The same study [7] also showed that the kinase protein and mRNA levels were low in the liver, and high in the kidney and heart, of rats fed a normal diet, which was correlated inversely with the activity state of the complex. These findings suggest that the BCOD kinase gene is subject to transcriptional regulation in response to nutritional stimuli in a tissue-specific manner.

In order to better understand hormonal effects on BCOD kinase gene expression, the hepatoma cell line H4IIE was chosen for study, because transcription of the phosphoenolpyruvate carboxykinase (PEPCK) was shown to be tightly regulated by cAMP, dexamethasone and insulin [12–14] in these cells. We show that the BCOD kinase mRNA is down-regulated by dexamethasone in H4IIE cells. However, the negative glucocorticoid-responsive element (GRE) is not located in the 3.0 kb kinase promoter region that was systemically investigated.

Abbreviations used: BCOD, branched-chain 2-oxoacid dehydrogenase; DMEM, Dulbecco's modified Eagle's medium; E1, branched-chain 2-oxoacid decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRE, glucocorticoid-responsive element; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisome-proliferator-activated receptor; TAT, tyrosine aminotransferase.

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Results obtained from studies in the rat indicate that the down-regulation of the kinase gene by dexamethasone is liver-specific. Possible mechanisms for the tissue-specific regulation of the BCOD kinase gene by hormonal and nutritional stimuli are discussed.

MATERIALS AND METHODS

Materials

The pPEPCK-CAT and pML-1 plasmids were kindly supplied by Dr. Robert Hammer (Department of Biochemistry, University of Texas Southwestern Medical Center) and Dr. Robert Makar (Department of Immunology, University of Texas Southwestern Medical Center) respectively. The pTAT plasmid was kindly supplied by Dr. John Koontz (University of Tennessee, Knoxville, TN, U.S.A.). α -Chloroisocaproate was generously donated by Dr. R. Simpson (Sandoz, Inc., East Hanover, NJ, U.S.A.).

Construction of promoter-luciferase vectors

A 6.5 kb *Bam*HI/*Sal*I (located at base +37 of the kinase promoter) fragment and a 3 kb *Bgl*II/*Sal*I fragment from the P1 plasmid [15] that hybridized with the radiolabelled primer 5'P₂ (5'-ACTGGGCACACACAGCC-3'; bases -33 to -17) were excised and purified using Qiaex resin (Qiagen, Chatsworth, CA, U.S.A.). To produce the 6.5 kb promoter construct (clone -6.5 K), the 6.5 kb fragment with the 5' end terminating in the -6.5 kb region was ligated to the *Bam*HI/*Sal*I-digested pBS-Luc PA vector. The 3 kb *Bgl*II/*Sal*I fragment and the 0.8 kb *Sal*I/*Pst*I fragment excised from promoter construct -33 were ligated into the *Bam*HI/*Pst*I-digested pBS-Luc PA vector. The remaining promoter constructs used in this study were described previously [15,16].

The 550 bp PEPCK promoter was excised from plasmid pPEPCK-CAT by *Bam*HI (base -550) and *Bgl*II (base +69) digestion. The PEPCK promoter fragments were ligated into the *Bam*HI-digested pBST-LucPA plasmid, which had been dephosphorylated using shrimp alkaline phosphatase. The orientation of the PEPCK promoter insert was determined by DNA sequencing.

To make promoter constructs for stable transformation, the pML-1 plasmid containing the neomycin-resistance gene driven by the simian virus 40 promoter was digested with *Xba*I, and made blunt-ended by Klenow fragment. The blunt-ended *Xba*I-cut vector was further digested with *Hind*III. A *Hind*III/*Spe*I fragment excised from the pBS-Luc PA plasmid containing the luciferase reporter gene was ligated into the previously digested pML-1 plasmid, generating the resultant plasmid pML-1-Luc. A *Bam*HI/*Sal*I fragment obtained from the kinase promoter constructs -3 K (described above), -449 or -58 (described previously [15,16]), with sizes of 3 kb, 490 bp or 100 bp respectively, and a 900 bp *Sal*I/*Hind*III fragment from plasmid -58, were ligated into pML-1-Luc vector previously digested with *Bgl*II and *Hind*III. The correctly inserted clone was determined by restriction enzyme digestion.

Cell culture, and transient and stable transfections

Rat hepatoma H4IIE cells (generously provided by Dr. C. R. Mendelson, Department of Biochemistry, University of Texas Southwestern Medical Center) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) calf serum. For hormonal studies, H4IIE cells were cultured in DMEM containing 10% (v/v) charcoal-treated fetal bovine serum (Cocalico Biologicals, Inc., Reamstown, PA, U.S.A.) and desired reagents or hormones.

To culture primary hepatocytes, approx. 3×10^6 viable cells were plated on a 100-mm culture dish coated with collagen type I from rat tail (Falcon). Cells were maintained in DMEM with 10% (v/v) charcoal-treated fetal bovine serum. At 2 h after plating, cells were re-fed with fresh medium.

Promoter constructs were transfected into H4IIE cells by electroporation, as previously described [16]. Electroporated cells were cultured on 60 mm-diam. dishes in a culture medium containing 10% (v/v) charcoal-treated fetal bovine serum and the desired reagents (dexamethasone, sodium butyrate or both) for 48 h before harvesting. Under these conditions, some cells that survived after electroporation did not carry plasmid cDNAs. Variations in transfection efficiency were corrected by normalization to co-transfected β -galactosidase activity.

To generate stably transformed cell lines, 10^7 H4IIE cells were electroporated at 1180 μ F/250 V with 15 μ g portions of pML-1-Luc plasmids carrying different lengths of the kinase promoter. Electroporated cells were incubated without selection for 24 h before the addition of 1 mg/ml G418. G418-resistant cells were isolated and expanded after 14 days. Transformed cells were treated or not with dexamethasone for 48 h before they were harvested for luciferase and β -galactosidase assays, as described previously [17].

Isolation of total and polyadenylated RNA, and Northern blot analysis

H4IIE cells cultured on 100 mm Petri dishes were rinsed with PBS and dissolved in 3 ml of an ice-cold lysis buffer containing 4 M guanidinium isothiocyanate, 1 mM EDTA and 25 mM sodium acetate. The lysate, sheared with syringe needles, was mixed with 20% (v/v) Sarkosyl (sodium lauryl sarcosinate) and layered on to a discontinuous 1 ml CsCl gradient (5.7 M CsCl, 1 mM EDTA and 25 mM sodium acetate) and centrifuged at 85000 *g* (Beckman SW-50.1) for at least 15 h. The CsCl-gradient-purified RNA was dissolved in diethyl pyrocarbonate-treated water, extracted twice with phenol/chloroform and precipitated with ethanol. Total RNA was isolated from tissues in acid guanidinium thiocyanate/phenol/chloroform, as described previously [18]. Total RNA isolated from primary hepatocytes was purified using an RNeasy® Kit (Qiagen), according to the manufacturer's protocol. Approx. 4×10^6 hepatocytes were dissolved in lysis buffer, and 50–80 μ g of total RNA was recovered using an RNeasy minicolumn. The amount of RNA was determined by measuring absorbance at 260 nm and 280 nm. Northern blot analysis was carried out as described previously [19].

DNA probe synthesis by hexamer random priming

A 700 bp *Bam*HI-digested BCOD kinase cDNA fragment, a 720 bp *Eco*RI/*Acc*I-restricted E1 α cDNA fragment and a 1.3 kb *Eco*RI-cut tyrosine aminotransferase (TAT) cDNA fragment were isolated from their respective host vectors, separated on agarose gels and purified with Qiaex resin (Qiagen). A 700 bp PEPCK partial cDNA fragment was synthesized by reverse transcriptase-PCR using the hepatic total RNA isolated from a dexamethasone-treated rat as template. Approx. 15 μ g of the liver total RNA and 100 pmol of an antisense primer (5'-CGTTTTCTGGGTTGATGGC-3') were used to synthesize the first-strand cDNA, as described previously [17]. The single-strand reaction product was used for PCR amplification using the same antisense primer and a sense primer (5'-ATGCCTC-CTCAGCTGCATAA-3'). The amplified 700 bp fragment was

purified and confirmed by DNA sequencing. The cDNA fragment was denatured at 95 °C for 2 min and chilled on ice. The denatured DNA was mixed with LS buffer (110 mM Tris/HCl, pH 8.0, 11 mM MgCl₂, 46 mM β -mercaptoethanol, 50 mM each of dGTP, dATP and dTTP, 0.43 M Hepes, pH 6.6, and 0.7 μ g/ μ l random hexamers [pd(N)₆ from Pharmacia]), 1 μ l of 10 mg/ml BSA, 100 μ Ci of [α -³²P]dCTP (specific radioactivity 3000 Ci/mmol) and 1 μ l of Klenow DNA polymerase (10 units/ μ l). After incubation at 37 °C for 2 h the ³²P-labelled DNA probe was purified from the free radiolabel by passage through a Sephadex G-50 spin column.

Animals and intraperitoneal injection

Sprague–Dawley rats used in this study were treated in accordance with guidelines set by the Animal Welfare Information Center and approved by the Institutional Animal Care and Use Committee of this institution. Male Sprague–Dawley rats were acclimated to a 12 h light/dark cycle and were fed *ad libitum* with chow or special formulated diets [20] for 14 days. Reagents were placed in a 1 ml syringe with hypodermic needle 25G 5/8. Rats were injected intraperitoneally with 1 mg of dexamethasone/day per 100 g body weight at 10:00 h daily for 3 days. Vehicle (saline)-injected rats served as controls. At 2 h after the final injection, animals were anaesthetized by inhalation of methoxyflurane, and tissues were collected by freeze-clamping in liquid nitrogen and stored at –70 °C.

Isolation of rat primary hepatocytes

The method was as described by Seglen [21], with modifications. Male adult rats (Sprague–Dawley) weighing approx. 200–250 g were used for hepatocyte isolation. Rats were anaesthetized by inhalation of methoxyflurane. The portal vein was incised and immediately perfused with 1 $\times$ Hanks solution, pH 7.4–7.5 (w/v: 0.8 % NaCl, 0.04 % KCl, 0.006 % KH₂PO₄, 0.035 % NaHCO₃, 0.009 % Na₂HPO₄·7H₂O, 0.1 % D-glucose and 0.001 % Phenol Red) at a rate of 40 ml/min for 5 min. The liver was then removed from the rat and placed in a bottle at 37 °C that was recirculated with 150 ml of 1 $\times$ Hanks solution containing 100 units/ml collagenase (Worthington) and 5 mM CaCl₂ at a rate of 40 ml/min for 10 min. The collagenase-perfused liver was cut into small pieces and suspended in RPMI 1640 medium to which 100 units/ml penicillin G sodium, 100 μ g/ml streptomycin sulphate and 0.2 mg/ml kanamycin sulphate were added. The suspension was passed through 310- μ m Spectra/Mesh, followed by centrifugation at 30 g for 2 min. Pelleted cells were resuspended in RPMI medium and passed sequentially through sieves of 310 μ m, 104 μ m and 75 μ m meshes, followed by centrifugation as described above. The pelleted cells were resuspended with RPMI medium and centrifuged several times until the supernatant was clear. The viability of isolated hepatocytes was examined by the Trypan Blue exclusion method.

Assays of actual and total enzyme activities of the BCOD complex

Cultured cells were harvested and washed twice with Kreb's buffer (0.7 % NaCl, 0.046 % KCl, 1.24 mM MgSO₄·7H₂O, 18 mM NaH₂PO₄, pH 7.4, and 0.0195 % CaCl₂). Washed cells were suspended in Kreb's buffer at a density of 2 $\times$ 10⁶ cells/0.1 ml (H4IIE cells) or 1 $\times$ 10⁶ cells/0.1 ml (primary hepatocytes). To assay actual activity, cells were rapidly frozen at –70 °C after harvesting. To assay total activity, cell suspensions were mixed with a 0.1 M stock of the kinase inhibitor α -chloroisocaproate [22], to give a final concentration of 1 mM, and incubated at

37 °C for 15 min. The cell suspension was rapidly frozen at –70 °C.

The assay mixture contained, in 295 μ l: 62.7 mM Tris/HCl, pH 7.5, 0.25 mM EDTA, 0.25 mM CoA, 0.44 mM MgCl₂, 0.25 mM NAD⁺, 0.25 mM thiamine pyrophosphate and 1.76 % (v/v) fetal bovine serum. To assay BCOD activity, 50 μ l of cell lysate or BSA solution (6 μ g/ μ l, as a background control) was added to the assay mixture. The mixture was kept on ice in one well of a 24-well plate sealed with Parafilm. The reaction was initiated by the injection of 25 μ l of 7.4 mM 5-oxo[1-¹⁴C]isovalerate (specific radioactivity 1500–1800 c.p.m./nmol). The resealed reaction mixture was incubated at 37 °C for 80 min, with the evolved ¹⁴CO₂ absorbed by an NaOH-soaked paper wick. To stop the reaction, 50 μ l of 15 % (v/v) trichloroacetic acid was injected into each well. The reaction mixture was incubated for additional 1 h at 37 °C to allow released residual ¹⁴CO₂ to be absorbed by the NaOH-soaked wick. The wicks were removed and radioactivity was counted in a Beckman scintillation counter. BCOD complex activity was determined by measuring the production of ¹⁴CO₂, which is the decarboxylation product of 5-oxo[1-¹⁴C]isovalerate. The specific activity of the BCOD complex was expressed as nmol of ¹⁴CO₂ released/min per mg of protein. The protein concentration was determined by the method of Lowry et al. [23] using BSA as a standard. The activity state (%) of the BCOD complex was expressed as: (actual activity)/(total activity) $\times$ 100.

RESULTS AND DISCUSSION

Repression of BCOD kinase transcription by dexamethasone in H4IIE cells

To investigate possible effects of prolonged glucocorticoid treatment on BCOD kinase gene expression, H4IIE cells were cultured in DMEM in the presence or absence of 100 nM dexamethasone for 30 h. Figure 1 shows that dexamethasone caused a marked decrease in the kinase mRNA level (lanes 4 and 6) compared with controls (lanes 3 and 5). The decrease in kinase mRNA content associated with dexamethasone treatment was not observed in FTO-2B (rat hepatoma) cells, which do not express a functional glucocorticoid receptor (Figure 1, lanes 1 and 2). Dibutyryl-cAMP and insulin were without effect on the kinase mRNA level in H4IIE cells during a 2-day culture (results not shown). The dexamethasone-induced decrease in the kinase mRNA level was accompanied by a 2-fold increase in the activity (dephosphorylation) state of the BCOD complex when H4IIE cells cultured for 48 h in the presence of the hormone were compared

Figure 1 Effects of dexamethasone on BCOD kinase expression in cultured H4IIE and FTO-2B cells

Cells cultured in medium with or without 100 nM dexamethasone (Dex) for 30 h and 48 h were used for isolation of total RNA and assays of BCOD complex activity respectively. The Northern blot shows levels of the kinase and E1 α mRNAs in FTO-2B (lanes 1 and 2) and H4IIE (lanes 3–6) cells cultured with (lanes 2, 4 and 6) or without (lanes 1, 3 and 5) 100 nM dexamethasone for 30 h.

Table 1 Effects of dexamethasone on the activity and activity state of the gBCOD complex in H4IIE cells

The actual and total activities of the gBCOD complex in H4IIE cells cultured in the presence or absence of 100 nM dexamethasone (Dex) for 48 h were measured as described in the Materials and methods section. The activity state (%) is defined as (actual activity)/(total activity) $\times$ 100. The data are expressed as means $\pm$ S.E.M. ($n = 3$)

Medium	Activity (nmol of $^{14}\text{CO}_2$ /min per mg of protein)		Activity state (%)
	Total	Actual	
DMEM	0.228 $\pm$ 0.036	0.110 $\pm$ 0.020	48.2 $\pm$ 201
DMEM + Dex	0.389 $\pm$ 0.071	0.373 $\pm$ 0.067	95.9 $\pm$ 3.3

Figure 2 Stability of BCOD kinase mRNA in H4IIE cells cultured in the presence or absence of dexamethasone

The stability of the kinase mRNA was measured in the presence of an mRNA synthesis inhibitor, actinomycin D (1 $\mu\text{g}/\text{ml}$). Total RNA (20 μg) isolated from cells cultured under different conditions at various time points was analysed by Northern blotting. Kinase mRNA levels were detected in the following samples: lane 1, control H4IIE cells; lanes 2–4 and lanes 5–7, H4IIE cells cultured in the presence and absence respectively of dexamethasone (Dex) (with actinomycin D); lanes 8–11 and 13, H4IIE cells treated with 0.1 μM dexamethasone for different periods of time (no actinomycin D); lanes 12, 13 and 14, H4IIE cells treated for 48 h with 0.01, 0.1 and 1 μM dexamethasone respectively (no actinomycin D). Probing of the same filter with an 18 S rRNA cDNA showed that approximately equal amounts of total RNA were applied to the agarose gel.

with untreated cells (Table 1). The total activity of the BCOD complex in dexamethasone-treated H4IIE cells was also increased by 70% over that in untreated cells. The induction of BCOD total activity was correlated with a slight increase in the E1 α mRNA level (Figure 1, lanes 3 and 5 compared with lanes 4 and 6). The combined effects of the increases in kinase and E1 α mRNA levels resulted in a 3-fold increase in actual BCOD activity in dexamethasone-treated compared with untreated H4IIE cells.

Induction of E1 α mRNA was previously observed in primary rat hepatocytes cultured in the presence of dexamethasone and a cAMP analogue, 8-(4-chlorophenylthio)-cAMP [24]. The same study also showed that the activity state of the BCOD complex in cultured hepatocytes was not affected by dexamethasone or the cAMP analogue, suggesting a lack of change in the kinase level. The basis for the conflicting results obtained with hepatoma cells (our Table 1) and cultured hepatocytes [24] is not entirely clear. However, some of the apparent contradictions are probably due to differences in culture conditions or the duration of the hormonal treatment. In the earlier report [24], cultured hepatocytes were incubated with dexamethasone for 24 h. Due to the relatively long half-life of the kinase mRNA (see below), down-regulation of this mRNA by dexamethasone is not readily

Figure 3 Effects of dexamethasone administration on the BCOD kinase mRNA level in rats fed on a protein-free diet

Rats were fed on protein-free diets for 14 days to induce the hepatic kinase mRNA, and then injected intraperitoneally with dexamethasone or a diluent control, as described in the Materials and methods section. Northern blot analyses of the BCOD kinase, PEPCK and GAPDH mRNAs and of 18 S rRNA were carried out. (A) Samples (20 μg) were of the total RNA from liver isolated from a rat fed on a chow diet (lane 1); rats fed on protein-free diets and then injected with diluents (lanes 2 and 3; duplicate samples from one rat); or identically fed rats injected with 1 mg of dexamethasone/day per 100 g body weight (lanes 4 and 5 from one rat; lanes 6 and 7 from another rat). (B) Total RNA isolated from different tissues, including heart (lanes 1–4), skeletal muscle (lanes 5–8), kidney (lanes 9–12) and spleen (lanes 13–16), was hybridized with the BCOD kinase and GAPDH probes. Samples in lanes 1, 5, 9 and 13 were isolated from one rat fed on a chow diet; samples in the other lanes were isolated from rats fed on protein-free diets. The total RNA isolated from rats injected with a diluent control (lanes 2, 6, 10, and 14) or dexamethasone (lanes 3, 7, 11 and 15 from one rat; lanes 4, 8, 12 and 16 from another rat) was analysed. Except for that isolated from skeletal muscle (10 μg), samples of 20 μg of total RNA isolated from the tissues were analysed by Northern blotting.

observed after a short incubation period. Moreover, culturing hepatocytes in hormone-free media often results in low basal gene expression for metabolic enzymes [25,26]. This may complicate studies on the repression of BCOD kinase gene expression by dexamethasone.

Although most genes regulated by dexamethasone show alterations in their transcription rates, the hormone can also affect the steady-state concentration of mRNA by changing its stability [27]. The reduced BCOD kinase mRNA level mediated by dexamethasone may have resulted from a down-regulation of the transcription rate or a decrease in kinase mRNA stability, or both. To differentiate between these possibilities, we estimated the apparent half-life of the kinase mRNA. This was accomplished by measuring the mRNA abundance as a function of time after addition of the transcription inhibitor actinomycin D (1 $\mu\text{g}/\text{ml}$) to H4IIE cells cultured in the presence (Figure 2, lanes 5–7) or absence (lanes 2–4) of dexamethasone. During the treatment period, neither cell death nor differences in cell morphology between control and dexamethasone-treated cells were observed. The maximal incubation time with actinomycin D was 24 h in this study, due to the toxic effects of actinomycin D on cells. The kinase mRNA level decreased during the incubation with actinomycin D in both untreated and dexamethasone-treated cells. The apparent half-lives of the kinase mRNA were estimated to be 16 h and 15 h in cells cultured with

Figure 4 Promoter activity of the BCOD kinase gene in H4IIE cells with or without dexamethasone treatment

DNA fragments representing various lengths of the promoter/regulatory region of the rat BCOD kinase gene were ligated into the pBST-Luc PA plasmid containing luciferase reporter cDNA. The promoter-reporter constructs were electroporated individually into H4IIE cells. (A) Constructs are designated according to the position of the 5'-terminal base in the DNA fragment. Except for constructs -6.5 K and -128/+37 (which have the 3'-end at base +37 in both clones), the 3'-end of all constructs is at base +264. The interrupted open box depicts intron 1 (581 bp), which interrupts the 5'-untranslated region (264 bp). (B) Electroporated cells were divided into portions and plated in four 60-mm dishes. Transfected cells were cultured in regular medium or the same medium containing 1 μ M dexamethasone (Dex, second bars), 2 mM sodium butyrate ('NaButyrate'), or both dexamethasone and sodium butyrate ('Dex + NaButyrate', fourth bars). To correct for variation in transfection efficiency, luciferase activity was normalized to that of co-transfected β -galactosidase. Luciferase activity was measured in cell extracts and expressed as relative light units/absorbance at 570 nm (β -galactosidase activity). pPEPCK-550 and pBST-Luc PA (Vector) served as a positive and a negative control respectively. Error bars are S.E.M. values ($n = 3-4$). (C) Promoter activity of H4IIE cells stably transfected with the kinase-promoter-luciferase constructs in the presence (□) or absence (■) of dexamethasone treatment. pML1-Luc kinase-promoter constructs, containing kinase-promoter regions of 3.5 kb, 449 bp or 58 bp in length, the luciferase cDNA and simian virus 40 transcribed neomycin-resistance gene were transfected into H4IIE cells by electroporation and selected in the presence of G418. Approx. 5×10^5 stably transformed cells were plated in 60-mm dishes and cultured in the presence or absence of 1 μ M dexamethasone. After a 48 h incubation, cells were harvested and luciferase activity was measured in cell extracts and expressed as relative light units/10³ cells. Error bars are S.E.M. values ($n = 3$).

(lanes 5-7) and without (lanes 2-4) dexamethasone respectively (Figure 2). The data are means of two measurements; only one of the two Northern blots is shown in Figure 2. The kinase mRNA in H4IIE cells was as stable as that of β -actin (results not shown), which explains why the down-regulation of the kinase mRNA by dexamethasone was only readily observed after 48 h of treatment (Figure 2, lanes 8-14). It is noteworthy that the effect of dexamethasone on the kinase mRNA was very sensitive, as it was observed at hormone concentrations as low as 10 nM (Figure 2, lane 12), which is below the physiological concentration of glucocorticoids. The equal loading of the total RNA was confirmed by the intensity of 18 S rRNA. These results established that the decreased steady-state level of the BCOD kinase mRNA was not caused by a decrease in mRNA stability.

Liver-specific regulation of BCOD kinase gene expression by dexamethasone

To investigate whether the effect of dexamethasone on BCOD kinase gene expression is physiologically significant, rats were fed on protein-free diets to induce the hepatic kinase mRNA, and then injected intraperitoneally with diluent (control) or dexamethasone (1 mg/day per 100 g body weight) for 3 consecutive days. The prolonged hormonal treatment *in vivo* took into consideration the relatively long half-life of the kinase mRNA measured *in vitro*. The rats were killed 2 h after the final injection, and the liver and other tissues were removed rapidly for RNA isolation. As shown in Figure 3(A), the hepatic kinase mRNA level was high in rats fed on a protein-free diet (lanes 2 and 3) compared with that in chow-fed rats (lane 1), and declined markedly in rats injected with dexamethasone (lanes 4-7), even when these rats were maintained on a protein-free diet. As a positive control, hepatic PEPCK mRNA was undetectable in rats fed on protein-free diets (Figure 3A, lanes 2 and 3), and increased sharply after dexamethasone injection (lanes 4-7), similar to the findings described previously [14,27-29]. Although the glucocorticoid receptor is ubiquitously expressed in tissues, the glucocorticoid-mediated decrease in BCOD kinase mRNA levels was only observed in the liver (Figure 3A), and not in the heart (lanes 1 and 2 compared with lanes 3 and 4), kidney (lanes 9-12), spleen (lanes 13-16) or skeletal muscle (lanes 5-8) from rats fed on protein-free diets (Figure 3B). This intact-animal study confirmed the down-regulation of the BCOD kinase mRNA level by dexamethasone observed in H4IIE hepatoma cells. More significantly, the present data suggest that the effect of dexamethasone on BCOD kinase gene expression is mediated through a liver-specific transcription factor(s). Transcriptional repression of the kinase gene was not due to a global down-regulation of basal transcription factors, since dexamethasone treatment did not alter the hepatic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level, which is not regulated by dexamethasone (Figure 3A).

An increased activity state of the BCOD complex was previously observed in skeletal muscle 2 h after injection of synthetic glucocorticoids [11]. In contrast, in the present study, the kinase mRNA level in the same tissue did not change after a 3-day treatment with the hormone. Wang et al. [30] recently reported that glucocorticoids increased the BCOD complex activity state from 67% to 82% in LLC-PK-GR101 cells. Our Northern blot analysis in collaboration with Price's group showed, however, that the kinase mRNA level in these cells was not affected in the presence of up to 200 nM dexamethasone (results not shown). The results imply that the hormone may exert its effects in skeletal muscle and renal cells through a post-translational inactivation of the kinase or an up-regulation of the phosphatase.

Figure 5 BCOD kinase mRNA levels in hepatocytes isolated from rats fed on chow or protein-free diets

(A) Hepatic polyadenylated RNA (2 μ g) isolated from rats fed on protein-free (0%; lanes 1 and 3, from two rats) or 50% (w/w) protein (lanes 2 and 4, from two other rats) diets for 14 days were probed with the kinase, PEPCK or TAT probes. (B) Hepatocytes isolated from chow-fed rats (lanes 1–5) or rats fed on a protein-free diet (lanes 6–10) were harvested at 2 h (lanes 1 and 6), 4 h (lanes 2 and 7), 8 h (lanes 3 and 8), 24 h (lanes 4 and 9) and 48 h (lanes 5 and 10) after plating. Total RNA was prepared from harvested hepatocytes. The levels of the kinase, PEPCK and TAT mRNAs and of 18 S rRNA at different times during the culture were analysed by Northern blotting.

Localization of the GRE in the BCOD kinase promoter

The finding that dexamethasone treatment did not affect the half-life of the BCOD kinase mRNA (Figure 2) strongly suggested that expression of the kinase gene was down-regulated at the transcriptional level. To identify the location of the putative negative GRE in the kinase promoter, an approx. 7 kb segment upstream of the translation start site, including the 6.5 kb promoter region, 581 bp of intron 1 and 264 bp of the 5'-untranslated region, was analysed by promoter-reporter assays in H4IIE cells cultured in the presence or absence of dexamethasone. The constructs used in the study are illustrated in Figure 4(A). As indicated in Figure 4(B), there was no obvious change in the kinase promoter activity in H4IIE cells with or without dexamethasone present. Likewise, dexamethasone was without effect on the promoter activities of the additional constructs -449 , -225 and -185 , all of which contained intron 1 and terminated at position $+264$ (results not shown). The absence of a response to dexamethasone was observed in the transient transfection assay even when the ratios of plasmid DNA to cell number were varied (results not shown). As a positive control, the PEPCK-550 promoter construct, which contained the GRE [14], showed a 10-fold induction of promoter activity in the presence of dexamethasone (Figure 4B). Results of the transient promoter assay indicate that the GRE is absent from the -3.0 kb region of the BCOD kinase gene promoter.

Although dexamethasone did not affect the promoter activity of the -6.5 K construct, deletion constructs between -3.0 and -6.5 kb need to be studied to establish that the GRE is not located in this region.

Inefficient DNA transfection into H4IIE cells was indicated by the low luciferase activity (1400–1800 light units) with the kinase promoter constructs (Figure 4B). It was therefore speculated that the repression of the kinase gene by dexamethasone may not have been readily observed. Several other DNA transfection methods were attempted, but none improved the efficiency of transfection of the kinase promoter constructs into H4IIE cells. To promote transcription of the reporter gene, sodium butyrate was added to the medium. This resulted in approx. 10-fold increases in kinase promoter activities compared with those measured in the absence of sodium butyrate. However, the luciferase activities in dexamethasone-treated and untreated cells cultured in the presence of sodium butyrate were again similar.

The transient promoter assays described above were rapid and free from interference by the chromosome positional effect; however, the results were complicated by the low transfection efficiency. We therefore established H4IIE cell lines containing stably integrated kinase promoter sequences (-3 K, -449 and -58). Extracts from 10^6 stable transformants were assayed for luciferase activity, which was normalized by the cell number (Figure 4C). There were no significant differences in luciferase activity in transformed cells cultured either with or without dexamethasone, similar to the results obtained with transient transfection. The stable transfection study confirmed that the negative GRE in the kinase gene is located either further upstream or downstream of the -3 kb region.

It was speculated that the inclusion of intron 1 in the promoter constructs may have complicated the promoter analysis. Both repressor and enhancer elements have been shown to be present in the first introns of many genes [31–35]. However, there were no significant differences in promoter activities between constructs $-128/+264$ and $-128/+37$, with and without intron 1 respectively (Figure 4B). The data indicate that *cis*-acting elements are absent from intron 1 located in the kinase promoter, and that inclusion of intron 1 would not affect the promoter assay. On the other hand, all constructs, with or without intron 1, exhibited luciferase activity. We interpret the data to indicate that intron 1 was correctly spliced out. This was confirmed by the absence from H4IIE cells of a 581 bp (the size of intron 1) longer kinase mRNA.

Induction of BCOD kinase mRNA during prolonged culture of primary hepatocytes

The levels of BCOD kinase mRNA are relatively high in H4IIE hepatoma cells and in livers from rats fed on protein-free diets, but low in livers from rats fed on a normal chow diet. This led to the hypothesis that transcription of the kinase in the liver is repressed under normal nutritional conditions, and that the kinase mRNA induced by protein malnutrition was the result of de-repression of kinase transcription. To approach this question, hepatocytes isolated from rats fed on protein-free or chow diets were cultured in DMEM without hormonal supplements. Figure 5(A) shows that the hepatic kinase mRNA level was markedly higher in rats fed on a protein-free diet than in those fed on a high-protein diet. In contrast, the level of TAT mRNA was much lower in rats fed on a protein-free diet than in those fed a 50% (w/w) protein diet. PEPCK mRNA was undetectable in rats fed on protein-free diets (Figure 5A, lanes 1 and 3), due to the high carbohydrate content of the diet, which will have repressed PEPCK gene transcription [36].

Table 2 Activity state and activity of the gBCOD complex in hepatocyte cultures

Hepatocytes isolated from rats fed on a chow diet or a protein-free diet were assayed for activity and activity state of the gBCOD complex after a 2 h or 24 h culture. The activity state (%) is defined as (actual activity)/(total activity) $\times$ 100. The data are expressed as means $\pm$ S.E.M. ($n = 3$).

Diet	Culture period (h)	Activity (nmol of $^{14}\text{CO}_2$ /min per mg of protein)		Activity state (%)
		Total	Actual	
Chow	2	0.461 $\pm$ 0.021	0.450 $\pm$ 0.026	97.04 $\pm$ 1.6
Chow	24	0.524 $\pm$ 0.070	0.197 $\pm$ 0.032	37.4 $\pm$ 1.5
Protein-free	2	0.532 $\pm$ 0.062	0.181 $\pm$ 0.010	34.2 $\pm$ 2.7
Protein-free	24	0.513 $\pm$ 0.031	0.190 $\pm$ 0.067	37.0 $\pm$ 3.1

It is noteworthy that, in hepatocytes isolated from rats fed on chow diets, the level of kinase mRNA gradually increased, whereas the levels of PEPCK and TAT mRNAs decreased as the culture time was prolonged up to 48 h (Figure 5B, lanes 1–5). In hepatocytes isolated from rats fed on protein-free diets, the relatively abundant kinase mRNA remained constant during the 48 h culture, whereas the PEPCK and TAT mRNAs were undetectable by Northern analysis (Figure 5B, lanes 6–10), similar to the results obtained with the whole liver (Figure 5A). The activity state of the complex was correlated inversely with the level of kinase mRNA. The activity state of the BCOD complex in hepatocytes isolated from chow-fed rats decreased from 97.4% to 37.4% when the culture period was increased from 2 h to 24 h (Table 2). In contrast, the activity state of the complex in hepatocytes from rats fed on protein-free diets remained constantly low (change from 34.2% to 37%) during the same culture period.

The differential BCOD kinase mRNA levels in cultured hepatocytes isolated from rats fed on chow diets strongly suggest the existence of factors *in vivo* in the liver that regulate kinase gene expression; these factors are postulated to be absent from the culture medium and other tissues. The trace amount of hepatic kinase mRNA present under normal nutritional conditions is probably caused by constitutive repression present in the liver. On the other hand, the 'induced' hepatic kinase mRNA in rats fed on protein-free diets may occur through inhibition of the repression cascade. We propose that the BCOD kinase gene is regulated in the liver by a repression/de-repression cycle imparted by glucocorticoids and nutritional stimuli. In support of this notion is the observation that DNA-binding activities and the levels of several liver-specific or -enriched transcription factors are decreased under protein-restricted conditions [37].

Concluding remarks

The discovery of retinoic acid receptors, retinoid X receptors [38] and peroxisome-proliferator-activated receptors (PPARs) [39–41] has led to the concept that nutrients play an important role in activating ligand-dependent transcription factors. For instance, retinoic acid and retinoid X receptors are activated by retinoic acids derived from vitamin A, and PPARs are activated by hypolipidaemic drugs or physiologically occurring ligands such as arachidonic acid and linoleic acid. PPAR α [39–41], which in turn activates several target genes involved in fatty acid catabolism, is subject to regulation by glucocorticoids [39,42].

Acyl-CoA oxidase in the β -oxidation pathway is a prototype enzyme whose expression is augmented by PPAR α [41] and dexamethasone. The latter hormone enhances the transcription of PPAR α ; however, the level of acyl-CoA oxidase mRNA targeted by PPAR α is not induced by dexamethasone in the absence of ligands such as WY-14643 [39,41]. These studies depict interesting interplays between metabolic hormones, ligand-dependent transcription factors and the target gene that encodes the enzyme involved in catabolism of the potential ligand fatty acids. Since the BCOD kinase gene is subject to tissue-specific regulation by glucocorticoids and nutritional stimuli, a similar mechanism may exist for regulating kinase gene expression in the liver. For example, clofibrate (*p*-chlorophenoxymethylpropionic acid), a well-known anti-hyperlipidaemic drug, was reported to increase hepatic BCOD-complex activity [43–45], which may be linked to the development of a muscular weakness syndrome after long-term treatment with the drug [46–48]. In a recent study [45], administration of clofibrate to chow-fed rats reduced the hepatic kinase mRNA and protein levels to 60% and 30% respectively of controls, which appeared to be responsible for the increased activity of the BCOD complex. Clofibrate can activate PPARs, and acetyl-CoA produced from catabolism of branched-chain amino acids is the precursor for fatty acid synthesis. In this context, clofibrate may serve as a ligand for the down-regulation of BCOD kinase gene expression by glucocorticoids through PPARs.

The present study shows that glucocorticoids decrease the steady-state level of the hepatic BCOD kinase mRNA, presumably by down-regulating transcription. Although H4IIE cells appear to be a good model system for studying the effects of dexamethasone on the regulation of the kinase, the use of transient transfection experiments was unable to identify the hormone-responsive element, which is likely to be located in the distal promoter region. A transgenic approach may be necessary to locate the GRE, liver-specific repressive elements and the elements that are regulated by nutritional stimuli in the kinase promoter. It will be interesting to demonstrate the cross-talk between these three elements in regulating BCOD kinase gene expression under various physiological conditions.

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REFERENCES

- Pettit, F. H., Yeaman, S. J. and Reed, L. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4881–4885
- Heffelfinger, S. C., Sewell, E. T. and Danner, D. J. (1983) *Biochemistry* **22**, 5519–5522
- Odessey, R. (1982) *Biochem. J.* **204**, 353–356
- Damuni, Z., Merryfield, M. L., Humphreys, J. S. and Reed, L. J. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4335–4338
- Harris, R. A., Paxton, R., Powell, S. M., Goodwin, G. W., Kuntz, M. J. and Han, A. C. (1986) *Adv. Enzyme Regul.* **25**, 219–237
- Harris, R. A., Powell, S. M., Paxton, R., Gillim, S. E. and Nagae, H. (1985) *Arch. Biochem. Biophys.* **243**, 542–545
- Harris, R. A., Paxton, R. and Jenkins, P. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 2463–2468
- Harris, R. A. and Paxton, R. (1985) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **44**, 305–315
- Harris, R. A., Paxton, R., Goodwin, G. W. and Powell, S. M. (1986) *Biochem. J.* **234**, 285–294
- Block, K. P., Heywood, B. W., Buse, M. G. and Harper, A. E. (1985) *Biochem. J.* **232**, 593–597
- Block, K. P., Richmond, W. B., Mehard, W. B. and Buse, M. G. (1987) *Am. J. Physiol.* **252**, E396–E407

- 12 Wynshaw-Boris, A., Lugo, T. G., Short, J. M., Fournier, R. E. K. and Hanson, R. W. (1984) *J. Biol. Chem.* **259**, 12161–12169
- 13 O'Brien, R. M., Lucas, P. C., Tomoyuki, T., Noisin, E. L. and Granner, D. K. (1994) *J. Biol. Chem.* **269**, 30419–30428
- 14 Hall, R. K., Sladek, F. M. and Granner, D. K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 412–416
- 15 Huang, Y. S. and Chuang, D. T. (1996) *Biochem. J.* **313**, 603–609
- 16 Huang, Y. S. and Chuang, D. T. (1998) *Biochem. J.* **334**, 713–722
- 17 Chuang, J. L., Cox, R. P. and Chuang, D. T. (1993) *J. Biol. Chem.* **268**, 8309–8316
- 18 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 19 Hu, C.-W., Lau, K. S., Griffin, T. A., Chuang, J. L., Fisher, C. W., Cox, R. P. and Chuang, D. T. (1988) *J. Biol. Chem.* **263**, 9007–9014
- 20 Popov, K. M., Zhao, Y., Shimomura, Y., Jaskiewicz, J., Kedishvili, N. Y., Irwin, J., Goodwin, G. W. and Harris, R. A. (1995) *Arch. Biochem. Biophys.* **316**, 148–154
- 21 Seglen, P. O. (1976) *Methods Cell Biol.* **13**, 29–83
- 22 Chuang, D. T. and Cox, R. P. (1988) *Methods Enzymol.* **166**, 135–146
- 23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- 24 Chicco, A. G., Adibi, S. A., Liu, W.-Q., Morris, S. M. and Paul, H. S. (1994) *J. Biol. Chem.* **269**, 19427–19434
- 25 Nebes, V. L. and Morris, S. M. (1988) *Mol. Endocrinol.* **2**, 444–451
- 26 Nebes, V. L. and Morris, S. M. (1987) *Biochem. J.* **246**, 237–240
- 27 Henderson, B. R. and Kefford, R. F. (1993) *Br. J. Cancer* **67**, 99–101
- 28 Yoo-Warren, H., Cimbala, M. A., Felz, K., Monahan, J. E., Leis, J. P. and Hanson, R. W. (1981) *J. Biol. Chem.* **256**, 10224–10227
- 29 Faber, S., O'Brien, R. M., Imai, E., Granner, D. K. and Chalkley, R. (1993) *J. Biol. Chem.* **268**, 24976–24985
- 30 Wang, X., Jurkovic, C. and Price, S. R. (1997) *Am. J. Physiol.* **272**, 2031–2036
- 31 Passantione, R., Antona, V., Barbieri, G., Rubino, P., Melchionna, R., Cossu, G., Feo, S. and Giallongo, A. (1998) *J. Biol. Chem.* **273**, 484–494
- 32 Wright, P. A. and Dobretsova, A. (1997) *Gene* **201**, 111–117
- 33 Antoine, M. and Kiefer, P. (1998) *Biochem. J.* **336**, 327–335
- 34 Storbeck, C. J., Sabourin, L. A., Waring, J. D. and Korneluk, R. G. (1998) *J. Biol. Chem.* **273**, 9139–9147
- 35 Gregori, C., Porteu, A., Lopez, S., Kahn, A. and Pichard, A.-L. (1998) *J. Biol. Chem.* **273**, 25237–25243
- 36 Meyer, S., Höppner, W. and Seitz, H. J. (1991) *Eur. J. Biochem.* **202**, 985–991
- 37 Marten, N. W., Sladek, F. M. and Straus, D. S. (1996) *Biochem. J.* **317**, 361–370
- 38 Mangelsdorf, D. J., Umesono, K. and Evans, R. M. (1994) in *The Retinoids: Biology, Chemistry and Medicine*, 2nd edn. (Sporn, M. B., Roberts, A. B. and Goodman, D. S., eds.), pp. 319–349, Raven Press, New York
- 39 Göttlicher, M., Widmark, E., Li, Q. and Gustafsson, J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 4653–4657
- 40 Boie, Y., Adam, M., Rushmore, T. H. and Kennedy, B. P. (1993) *J. Biol. Chem.* **268**, 5530–5534
- 41 Lemberger, T., Saladin, R., Vázquez, M., Assimacopoulos, F., Staels, B., Desvergne, B., Wahli, W. and Auwerx, J. (1996) *J. Biol. Chem.* **271**, 1764–1769
- 42 Lemberger, T., Staels, B., Saladin, R., Desvergne, B., Auwerx, J. and Wahli, W. (1994) *J. Biol. Chem.* **269**, 24527–24530
- 43 Ono, K., Shioya, H., Hakozaaki, M., Honda, K., Mori, T. and Kochi, H. (1990) *Biochem. Biophys. Res. Commun.* **172**, 243–248
- 44 Zhao, Y., Jaskiewicz, J. and Harris, R. A. (1992) *Biochem. J.* **285**, 167–172
- 45 Paul, H. S., Liu, W. Q. and Adibi, S. A. (1996) *Biochem. J.* **317**, 411–417
- 46 Paul, H. S. and Adibi, S. A. (1980) *J. Clin. Invest.* **65**, 1285–1293
- 47 Pardridge, W. M., Casanella-Ertl, D. and Duducgian-Vartavarian, L. (1980) *J. Clin. Invest.* **66**, 88–93
- 48 Danner, D. J., Sewell, E. T. and Elsas, L. J. (1982) *J. Biol. Chem.* **257**, 659–662

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