Glucocorticoid regulation of branched-chain α -ketoacid dehydrogenase E2 subunit gene expression

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Regulation of the mammalian branched-chain α -ketoacid dehydrogenase complex (BCKAD) occurs under a variety of stressful conditions associated with changes in circulating glucocorticoids. Multiple levels of regulation in hepatocytes, including alteration of the levels of the structural subunits available for assembly (E1, α -ketoacid decarboxylase; E2, dihydrolipoamide acyltransferase; and E3, dihydrolipoamide dehydrogenase), as well as BCKAD kinase, which serves to phosphorylate the E1 α subunit and inactivate complex activity, have been proposed. The direct role of glucocorticoids in regulating the expression of the murine gene encoding the major BCKAD subunit E2, upon which the other BCKAD subunits assemble, was therefore examined. Deletion analysis of the 5' proximal 7.0 kb of the murine E2 promoter sequence, using E2 promoter/luciferase expression minigene plasmids introduced into the hepatic H4IIEC3 cell line, suggested a promoter proximal region responsive to glucocorticoid regulation. Linker-scanning muta-

genesis combined with deletion analysis established this functional glucocorticoid-responsive unit (GRU) to be located near the murine E2 proximal promoter site at –140 to –70 bp upstream from the transcription initiation site. The presence of this region in plasmid minigenes, containing varying amounts of the murine genomic sequence 5' upstream from proximal E2 promoter sequences, conferred 2–10 fold increases in luciferase reporter gene expression in H4IIEC3 cells, whether introduced by transient transfection or following co-selection for stable transfectants. The GRU region itself appeared to contain multiple interacting elements that combine to regulate overall E2 promoter activity in response to changing physiological conditions associated with varying concentrations of glucocorticoids and likely other hormonal effectors.

Key words: BCKAD E2, glucocorticoid response unit, promoter.

INTRODUCTION

The mitochondrial enzyme complex, branched-chain α -ketoacid dehydrogenase (BCKAD, EC 1.2.4.4), catalyses the rate-limiting step of branched-chain amino acid catabolism. Through the oxidative decarboxylation of the branched-chain α -ketoacids derived from the branched-chain amino acids leucine, isoleucine, and valine, the BCKAD complex directs their metabolic flow away from protein biosynthesis and towards the production of gluconeogenic and ketogenic substrates required to meet changing energy requirements. The BCKAD complex is composed of α -ketoacid decarboxylase (E1, EC 1.2.4.4), dihydrolipoamide acyltransferase (E2, no EC number), and dihydrolipoamide reductase (E3, EC 1.8.1.4) activities which catalyse the overall oxidative decarboxylation. In addition, BCKAD kinase (EC 2.7.1.115) and phosphatase activities are associated with the complex, providing a mechanism of regulation through phosphorylation (inactivation) and dephosphorylation (activation) of the E1 α -subunit proteins. The BCKAD complex appears to be composed of 24 E2-subunit proteins upon which 24 E1 α and E1 β proteins (associated as 12 $\alpha_2\beta_2$ subunits), 6 E3 dimers, an undetermined number of BCKAD kinase and phosphatase molecules, and several bound cofactors assemble [1,2].

A large number of studies have demonstrated that there are many influences on BCKAD activity. Changes in BCKAD activity may be produced in response to endocrine factors,

exercise, and nutritional state through post-translational mechanisms of regulation, such as the kinase-mediated phosphorylation (inactivation), as well as through pre-translational mechanisms which lead to increases in the RNAs which encode the BCKAD-subunit proteins [3,4]. Both types of regulatory mechanisms appear to be used in mammalian liver, the organ demonstrating the highest activity per organ and the majority of the total body BCKAD capacity in laboratory rats [5]. A number of laboratories have examined the responses of hepatic BCKAD activity to changes in dietary protein and caloric intake [5–19]. Examination of steady-state levels of RNAs encoding BCKAD subunits (E1 α , E1 β , E2, and kinase) in rodent tissues obtained from animals at different post-natal developmental ages or fed on diets with various protein and/or caloric contents, suggested that regulation of BCKAD-subunit gene expression plays an important role in these responses [6,16–21]. Several developmental and nutritional states which are associated with elevated levels of glucocorticoids (e.g. caloric starvation or high dietary protein), as well as direct testing of glucocorticoids on primary hepatocytes, have been associated with increases in the levels of E2-subunit RNA and protein, the subunit on which the others assemble [6,16–19,22]. In some cases, changes in E2-RNA levels were not coordinated with changes in E1 α -subunit RNA levels, suggesting the potential for mechanisms allowing independent regulation of the expression of BCKAD-subunit genes [17–19]. In this report, we present our studies focusing on the

Abbreviations used: BCKAD, branched-chain α -ketoacid dehydrogenase complex; E1, α -ketoacid decarboxylase; E2, dihydrolipoamide acyltransferase; E3, dihydrolipoamide dehydrogenase; DEX, dexamethasone; dBcAMP, dibutyl cAMP; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; FCS, fetal calf serum; GRU, glucocorticoid-responsive unit; RSV; Rous sarcoma virus; SEAP, secreted alkaline phosphatase

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regulation of murine BCKAD-E2 promoter sequences by glucocorticoids. Our results suggest that a glucocorticoid-response unit (GRU) is present near the transcriptional start site and may contribute to a potential 5–10 fold increase in BCKAD-E2 promoter activity, more than adequate to account for the range of steady-state RNA levels observed in rodent tissues under different dietary and developmental conditions.

EXPERIMENTAL

Chemicals

Unless otherwise specified, all chemicals were purchased from (Sigma). Restriction endonucleases, other modification enzymes, and all cell culture media were purchased from Life Technologies (Rockville, MD, U.S.A.), unless otherwise noted. Fetal calf serum (FCS) was obtained from BioWhittaker (Walkersville, MD, U.S.A.). Luciferase assay system and pGL2-promoter luciferase expression vectors were obtained from Promega (Madison, WI, U.S.A.). Phosphalight secreted alkaline phosphatase (SEAP) assay system was purchased from Tropix (Bedford, MA, U.S.A.).

Cell culture

Rat hepatoma H4IIEC3 cells were obtained from the A. T. T. C., and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS at 37 °C in humidified 5% CO₂, 95% air atmosphere. Specific experimental conditions are indicated in the Figure legends. Cells were plated in serum containing medium for 24 h, washed twice with PBS, then fed with serum-free medium for 14–16 h. Following the addition of dexamethasone (DEX, 0.001–1.0 μ M) and/or dibutyryl cAMP (dBcAMP, 0.1–1.0 mM) cells were incubated in fresh serum-free media for the indicated time interval prior to harvest for BCKAD RNA or reporter gene analysis. Minigene transfection experiments utilized 1.0 μ M DEX and 0.5 mM dBcAMP, unless otherwise specified. Cells were prepared for RNA analysis as previously described [18,19,23,24]. For transfections, cells were multiply-plated from a single batch of freshly trypsin-treated cells, at 3.5×10^5 cells per 20 mm well into 6-well cell culture dishes (Falcon).

Minigene plasmids

The isolation, cloning and sequencing of the murine BCKAD E2 promoter region, containing 7.0 kb 5' upstream genomic sequence and downstream luciferase reporter sequence (pGL-Basic luciferase vector; Promega), is described elsewhere (GenBank accession number U95963) [27]. Available restriction sites in the promoter and 5' upstream genomic region were used in a series of restriction endonuclease and religation steps to obtain sequentially smaller BCKAD E2 gene sequences ranging from 7.0 kb to 300 bp from the full-sized 7.0 kb pGLE2-7.0 minigene plasmid, as previously described [27]. The restriction enzymes used in their creation included: pGLE2-4.0, *Kpn*I; pGLE2-2.3, *Spe*I; pGLE2-0.9, *Sac*I; pGLE2-0.3, *Pst*I. Clones containing 5' upstream regions less than 300 bases in length were obtained by PCR amplification of the region desired, followed by restriction-enzyme digestion and ligation. The end sequences of the larger minigenes as well as the entire sequence of the smaller minigenes were confirmed by use of the double-stranded DNA Cycle Sequencing System (Life Technologies) as well as cycle sequencing on the Applied Biosystems Automated Sequencer (Model 373A; Applied Biosystems, Foster City, CA, U.S.A.). All of the BCKAD E2 sequences terminate at the BspE2 sequence (TCCGGA) 6–11 bp upstream from the initiation ATG codon in

exon 1, and 8 bp downstream from the determined BCKAD E2 transcription initiation site [27]. The cloned 90 bp promoter region of the human thromboxane synthase gene (TX90) was provided by the laboratory of R. F. Shen and cloned into the luciferase expression vector pGL Basic, as described in [28]. pRous sarcoma virus (RSV)-SEAP, a minigene plasmid which expresses a secretory form of alkaline phosphatase, was obtained from Tropix.

Transient transfection of H4IIEC3 cells

H4IIEC3 cells were plated in DMEM containing 10% FCS, incubated overnight, washed with PBS, and replaced with DMEM without FCS. A 100 μ l aliquot of DMEM containing plasmid DNA (0.4 μ g of RSV-SEAP/1.6 μ g of test BCKAD minigene) was mixed with 100 μ l of media containing 8 μ l of Lipofectamine (Life Technologies), and incubated at room temperature for 30 min. The optimal amounts of Lipofectamine and plasmid needed to obtain highest transfection efficiency were determined by systematic titration of both parameters and determination of transfection efficiency based on levels of SEAP in the media (results not shown and [27]). After 800 μ l of DMEM was added to the suspension, the cells were overlaid with the mixture and incubated for 4 h (37 °C, 5% CO₂). The plates were washed twice with PBS and DMEM/10% FCS was added. After overnight incubation, the cells were washed twice with PBS to remove dead cells and media replaced with fresh DMEM/10% FCS. Cells were incubated for 8–10 h, then media was collected for SEAP analysis to assess relative transfection efficiency. The cells were washed twice with PBS and FCS-free DMEM was added. After overnight incubation, the media was replaced with fresh FCS-free DMEM containing DEX and/or dBcAMP and the cells incubated for the indicated times (usually 24 h), and harvested for determination of luciferase activity.

Production and analyses of stably transfected cell lines

H4IIEC3 cells were transfected with DNA in a 1:4 ratio of a neomycin-expression plasmid under the control of an RSV promoter (RSV-Neo) to a cloned luciferase expression plasmid (pGL2 Basic; Promega), under the control of inserted BCKAD E2 genomic promoter sequence (pGLE2-7.0 or pGLE2-0.3) [27]. Cells were plated at 2×10^5 cells per 60 mm dish, incubated overnight in DMEM/10% FCS, washed with PBS, and then covered with 5 ml of DMEM containing 8% modified bovine serum (Stratagene, La Jolla, CA, U.S.A.). Then, 10 μ g of plasmid DNA was mixed in 450 μ l of water with 50 μ l of 2.5 M CaPO₄ and 500 μ l of 2 $\times$ buffered saline (pH 6.95), and the suspension incubated at room temperature for 10–20 min and added to the cells dropwise (according to manufacturer's recommendations; Stratagene). After 3 h incubation at 35 °C, 3% CO₂, the cells were washed 3 times with PBS, covered with DMEM/10% FCS, and incubated at 37 °C, 5% CO₂. After overnight incubation, the cells were washed to remove dead cells and the media replaced with fresh DMEM/10% FCS. The following day, the media was supplemented with 400 μ g/ml of G418 (Geneticin; Life Technologies) and maintained under this selection for 3–4 weeks with the media replaced every 4 days. Colonies formed by neomycin-resistant cells were individually trypsin-treated and plated separately, or in pools. Upon expansion, the cells were assayed for luciferase activity and frozen under liquid nitrogen in 10% DMSO/20% FCS.

When used, frozen colonies were thawed, passaged several times to ensure high viability, and then tested as a single batch for comparative analyses. Cells were plated at 3.0×10^5 cells per 20 mm well in 6-well cell culture dishes, incubated overnight in

DMEM/10% FCS, washed twice with PBS, and incubated overnight (14–16 h) with DMEM without FCS. The media was then replaced with FCS-free DMEM containing DEX and/or dBcAMP, and the cells incubated for 24 h when the cells were harvested for determination of luciferase activity. Analyses using a singly cloned cell line containing pGLE2-7.0, H4S1, are presented here. Analyses using other independently cloned cells lines were performed to confirm the results reported for H4S1.

Reporter gene assays

SEAP activity was measured from culture medium (100 μ l) collected from the transfected cells, according to the directions of the assay kit supplier (Tropix). This media was mixed with 1x dilution buffer (300 μ l), heated to 65 °C for 30 min, then 100 μ l of heat-treated media was mixed with 100 μ l of assay buffer containing a mixture of different alkaline phosphatase inhibitors. These inhibitors allow the detection of the secreted placental alkaline phosphatase, while minimizing the interference from non-heat inactivated endogenous phosphatase activities. The reaction buffer containing CSPD® (Tropix) chemiluminescent substrate (100 μ l) was added to the reaction tube and after 20 min incubation at room temperature, the luminescence was measured with a Turner Designs Model 20e luminometer (Turner Designs, Sunnyvale, CA, U.S.A.).

Luciferase activity was measured according to the directions of the assay kit supplier (Promega). Transfected cells were washed twice with PBS and covered with 200 μ l (per 20mm well) of 1x reporter lysis buffer, incubated for 15 min at room temperature, and scraped into a 1.5 ml microfuge tube. Following vortex mixing for 10 s and microfuge centrifugation at 12000 *g* for 10 s, 20 μ l of cell extract was mixed with 100 μ l of luciferase assay reagent (Promega) and luminescence determined with the Turner Designs Model 20e luminometer.

All assay values were measured in duplicate or triplicate to ensure proper values. All transient transfections were performed in triplicate in the same multiwell dish, and similarly, all stably transfected cells were assayed in triplicate. Luciferase activity from each transiently transfected sample was corrected for SEAP activity in media collected prior to serum starvation and exposure of the cells to DEX/dBcAMP. The ratio of the luciferase/alkaline phosphatase activities from the triplicate samples was averaged and the Student's *t*-test performed based on the mean and the S.D.

Gel-retardation (gel-shift) assay

Nuclear extracts were prepared from H4IIEC3 cells and used in gel-retardation assays, as described by Berg et al., using standard protocols described elsewhere [29,36,37]. Following growth to confluency (5 $\times$ 150 mm dishes), H4IIEC3 cells were scraped and washed twice in cold PBS, resuspended and swelled on ice for 10 min in 5 ml of 10 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol (DTT), and 0.5 mM PMSF, then centrifuged and lysed in fresh buffer containing 0.05% (v/v) Nonidet P40 using a Dounce homogenizer. The pellet was recentrifuged and resuspended in 2 ml of 5 mM Hepes (pH 7.9), 1.5 mM MgCl₂, 0.2 mM EDTA, 26% glycerol, 0.5 mM DTT and 0.5 mM PMSF, to which concentrated NaCl was added to make the final solution 420 mM, which was then incubated on ice for 30 min. The resulting lysate was centrifuged at 24000 *g* for 20 min in an Sw41 Beckman rotor, dialysed through a 0.0025 μ m VS Millipore membrane (Millipore) against 5 mM Hepes (pH 7.9), 100 mM KCl, 0.2 mM EDTA, 26% glycerol, 0.5 mM DTT, and 0.5 mM PMSF at 4 °C, and frozen in 50 μ l aliquots in liquid nitrogen for use for individual experiments. For the gel-

tardation assays, oligonucleotide DNA prepared from the murine BCKAD E2 promoter region by PCR amplification and gel purification (–47 to –140 bp upstream from the start site for transcription) was end-labelled with [γ -³²P]ATP by T4 polynucleotide kinase, mixed with approx. 3 μ g of nuclear extract, incubated at room temperature for 20 min, and loaded in 1 $\times$ gel-shift binding buffer [4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris (pH 7.0), 0.05 mg/ml poly(dI-dC)] onto a 5% non-denaturing polyacrylamide gel containing 2.5% glycerol. After electrophoresis at 100 V for 3 h in 0.5 $\times$ TBE, the gel was dried under vacuum and exposed to X-ray film at –80 °C.

RESULTS

Glucocorticoids affect BCKAD E2 promoter activity in stably transfected H4IIEC3 cells

The established rat hepatoma H4IIEC3 cell line has been previously shown to demonstrate consistent changes in BCKAD activity and subunit RNA in response to the presence of glucocorticoids [19,21,29]. Following exposure to DEX under a variety of conditions, consistent increases in steady state RNA levels (2–3 fold) are observed for the BCKAD E2 subunit but not the E1 α subunit [19,29]. In some cases, the addition of dBcAMP with DEX will enhance the apparent glucocorticoid response, similar to the observations in other glucocorticoid-responsive genes [29,30]. These cells were therefore used to determine the effects of glucocorticoids on E2 BCKAD promoter activity.

Using an E2 minigene plasmid (pGL-E2 7.0 kb) containing 7.0 kb of 5' murine genomic sequence, with promoter activity directing expression of the reporter gene luciferase, stably transfected H4IIEC3 cells were isolated by selection for resistance to the neomycin analogue, G418, based on co-transfection with a neomycin-resistance gene expression plasmid. Independently cloned cell lines expressing luciferase were isolated and tested for their responsiveness to DEX and DEX plus dBcAMP. Since individual transfected clones could respond differently based on a regional effect at the sites of recombination of the transfected plasmids, all of the responses observed with one stable cell line were confirmed with other independent stably transfected cloned cells and also with transient transfection studies (see below). All of the studies reported below were with the cloned stably transfected cell line H4S1. The effect of DEX and dBcAMP singly and in combination on H4S1 was examined (Figure 1). Similar to the results observed with RNA levels, exposure to DEX produced 2-fold increases in luciferase, dBcAMP alone produced no increases, and in combination, DEX/dBcAMP produced about 3–4 fold increases in luciferase activity within 24 h. Further increases were observed at 48 h, up to 6–7 fold in the DEX/dBcAMP treated cells (results not shown). Titration analysis of DEX exposure of H4S1 cells demonstrated increases in relative promoter activity with 0.001–1.0 μ M DEX in the presence of a constant amount of dBcAMP known to augment the DEX response (results not shown). To confirm the involvement of a glucocorticoid-receptor mediated mechanism in the activation of the E2 gene, we treated H4S1 cells with a combination of DEX and the glucocorticoid receptor antagonist, RU486 (Roussel Uclaf, [30]). Treatment of the cells with RU486 in combination with DEX prevented the DEX-associated activation of the E2 promoter activity (results not shown). This finding suggested that the glucocorticoid receptor participated in the regulation of E2 by glucocorticoids, but did not indicate a direct interaction of the glucocorticoid receptor with the E2 promoter sequence.

Figure 1 Effect of glucocorticoids on BCKAD E2 7.0 kb minigene promoter activity in stably transfected H4IIEC3 cells

The cloned isolate of H4S1 cells were plated in triplicate, allowed to grow in the presence, then absence, of FCS, then exposed to dexamethasone (DX, 1.0 μ M) and dBcAMP (dB, 1.0 mM) for the indicated times and harvested for luciferase activity as described in the Experimental section. Relative promoter activity is given as the relative percent change in luciferase (LUC) luminescence units (L.U.) observed compared to basal promoter activity in the absence of glucocorticoids, whose value is set at 100% (Ctrl, control luciferase). All values represent the means $\pm$ S.D. of three samples. All increases above baseline more than 2-fold (200%) were statistically significant ($P < 0.05$). When cells were exposed to glucocorticoids for 48 h, 3–4 fold increases with DEX and 5–6 fold increases with DEX + dB were observed (results not shown).

Regional localization of the sequences involved in glucocorticoid regulation of BCKAD E2 promoter activity

To identify the region that contained *cis*-acting elements responsible for the glucocorticoid-mediated activation of the BCKAD E2 promoter, a series of luciferase expressing minigenes, containing decreasing amounts of E2 genomic DNA 5' to the transcription start site, were assayed by transient transfection into H4IIEC3 cells [27]. We had previously determined that BCKAD promoter activity decreased when the included 5' upstream region decreased from 7.0 kb to 0.3 kb of genomic material [27]. In contrast, the relative promoter activity in response to the presence of DEX or DEX and dBcAMP increased up to 5–10 fold (Figure 2). These findings suggested that the proximal promoter 0.3 kb region contained sequences sufficient for glucocorticoid-mediated activation.

To further confirm the glucocorticoid responsiveness of the smaller BCKAD sequences, stably transfected H4IIEC3 cloned cell lines were obtained following transfection with the smaller BCKAD minigenes. Mean relative promoter activity increased 2–3 fold in the cells transfected with either the 0.9 or the 0.3 kb E2 minigene when measured in pooled cell samples (results not shown). In the cloned cell line 0.3S3, derived from selection following transfection with the minigene containing 315 bp of E2 upstream promoter sequence, relative luciferase activity increased by $218 \pm 6\%$ ($n = 3$). This 2–3 fold increase in promoter activity in response to glucocorticoids was blocked in the presence of RU486. For example, luciferase/SEAP ratios changed from 19.38 ± 0.65 to 41.78 ± 1.73 in the presence of 1 μ M DEX, a change of 2.16-fold that was blocked in the presence of 1 μ M RU486 (from 13.25 ± 0.46 to 16.31 ± 0.23 ; $n = 3$), similar to

Figure 2 Effect of deletion of 5' genomic sequences on the activity and glucocorticoid responsiveness of the BCKAD E2 promoter region

A series of BCKAD E2 promoter minigenes were constructed containing increasing contiguous regions of the 5' upstream genomic sequences deleted from the parent E2–7.0 kb minigene. The basal activity of each minigene was tested by transient transfection into H4IIEC3 cells ($n = 3$) and measurement of resultant luciferase activity standardized to the amount of alkaline phosphatase activity obtained following cotransfection of a SEAP-containing minigene (means $\pm$ S.D.), as described in the Experimental section. The effect of the addition of dexamethasone (1.0 μ M) and dBcAMP (1.0 mM) to these cells following transfection is shown. Promoter activity is given as the relative percentage change in luciferase (LUC) luminescent units/SEAP luminescent units observed following addition of glucocorticoid compared to untreated transfected cells (% Control LUC/SEAP is set at 100% promoter activity under basal, or untreated, conditions for each individual plasmid transfected). No statistically significant effect of dBcAMP was noted with these minigenes when added in the absence of DEX. All increases above baseline more than 2-fold (200%) were statistically significant ($P < 0.05$).

Figure 3 Regional localization of glucocorticoid-responsive sequences

E2 promoter reporter minigenes were prepared containing the indicated number of base pairs located 5' upstream from the E2 transcription start site. Following transfection of H4IIEC3 cells ($n = 3$ for each), SEAP activity was determined and then luciferase activity determined following the addition (1.0 μ M) or absence of DEX. One of several heterologous promoter sequences (murine thromboxane synthase, TX 90) was used as a control for nonspecific effects of DEX on cell metabolism. The LUC/SEAP activities ratios are indicated as the relative values observed 20 h following DEX addition (100% indicates no change in promoter activity) compared to the baseline activities determined in the absence of DEX for each minigene tested. Each value represents the means $\pm$ S.D.

Figure 4 Sequence and putative transcription-factor-binding sites of the BCKAD E2 promoter region

The region of the minimal E2 promoter, including the first exon (in contiguous **bold** capital letters, +1-- +64), translation initiation site (ATG, double underlined), transcriptional start site (+1 'A' marked with top horizontal line), and putative transcription factor sites (underlined) identified using the programs of sequence analysis available through the Wisconsin Package, were previously determined [27]. The bases underlined in the GRE and HNF-3 indicate identity with the E2 genomic sequence (11–12/15 and 8–9/12 respectively). SDR refers to the octanucleotide site identified in sterol-dependent protein repression of activity in several genes [35]. The restriction endonuclease site BspE1, used for cloning into pGL2Basic to allow expression of a downstream luciferase gene, is indicated (+8 site of ligation, underlying line).

-140	SDR/R	AP2	CAAT/R	-70...+8	PLASMID	ACTIVITY
GGAC	<u>CACCACCCCTCCCCCTGGTT</u>	<u>GGCTGCCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG</u>	..+8	pGL53	100±6	
	pyrimidine	pyrimidine				
GGACA	<u>AAGAGCTCA</u>	CCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGR1	84±8	
GGACACC	CCCCCTCCCC <u>AGAGCTCA</u>	AGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGR2	51±4	
GGACACC	CCCCCTCCCCCTGGTTG <u>AGAGCTCA</u>	AGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGR3	110±11	
GGACACC	CCCCCTCCCCCTGGTTGGCTGCCCG <u>AGAGCTC</u>	CGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGR4	81±9	
GGACACC	CCCCCTCCCCCTGGTTGGCTGCCCGGTGGGAGG <u>AGAGCTCA</u>	TTCTCTCCTATTGGCCGCTCAGG	..+8	pGR5	138±6	
GGACACC	CCCCCTCCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTT <u>GAGCTC</u>	TATTGGCCGCTCAGG	..+8	pGR6	51±4	
GGACACC	CCCCCTCCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCT <u>GAGCTCT</u>	GCTCAGG	..+8	pGR7	33±3	
GGACA	<u>AAGAGCTCA</u>	CCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCT <u>GAGCTCT</u>	GCTCAGG	..+8	pGR71	12±2
GGACACC	CCCCCTCCCC <u>AGAGCTCA</u>	AGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCT <u>GAGCTCT</u>	GCTCAGG	..+8	pGR72	13±3
GGACACC	CCCCCTCCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	PGL53	100±15		
-127	TCCCCCTGGTTGGCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGRL1	90±7		
-116	GCTGCCCGGTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGRL2	74±16		
-108	GTGGGAGGCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGRL3	69±10		
-101	GCTCGTTCCTTCTCTCCTATTGGCCGCTCAGG	..+8	pGRL4	74±13		
-92	TTCTCTCCTATTGGCCGCTCAGG	..+8	pGRL5	44±7		
-85	CTATTGGCCGCTCAGG	..+8	pGRL6	19±2		
-76	GCTCAGG	..+8	pGRL7	9±2		

Figure 5 Linker scanning and deletion mutagenesis of the BCKAD E2 glucocorticoid response unit sequences

Promoter sequence region modified by site-directed mutagenesis is shown on top line for parent minigene pGL53 (–140 to +8 bp E2 sequence ligated to pGLBasic luciferase expression vector) with putative transcription-factor-binding sites or other regions of interest indicated (underlined and **bold** on first line). Modified minigene sequences are listed (pGR1–GR7, single substitution and pGR71 and 72, double substitution) with the substituted bases (**bold** and underlined) created within the *SacI*-containing linker (GAGCTC). Deleted minigenes listed (pGRL1–7) derived from *SacI* restriction digest and religation are shown below. The inserted size in each construct was confirmed by PCR amplification using flanking oligonucleotide primers and analysis on agarose gel electrophoresis. The relative basal promoter activities of each of the modified minigenes compared to the parent minigene pGL53 (set at 100%) for two separate experiments (top and bottom series) are listed ($n = 3$, means $\pm$ S.D.) in the right column (see the Experimental section).

the results obtained with the H4IIEC3 cells stably transfected with the entire 7.0 kb E2 sequence (H4S1 cells described above).

To narrow down the critical promoter region containing sequences needed for the DEX-associated glucocorticoid effect, a series of minigenes, with progressively shorter regions of the BCKAD promoter DNA (from 315 to 44 bp upstream from the transcription initiation site), were assayed (Figure 3). Comparison of these relative increases in promoter activity in the presence of DEX suggested that sequences within the first 140 bp upstream from the transcriptional start site contained *cis*-acting

elements sufficient to mediate glucocorticoid activation of the E2 promoter. Luciferase activity in cells transfected with the 140 bp minigene plasmid was 5-fold higher in DEX-treated than control, unexposed cells. The activity of the heterologous eukaryotic thromboxane synthetase promoter failed to increase when these transfected cells were exposed to DEX (Figure 3). This suggested that the glucocorticoid activation of the E2 promoter was specific and not secondary to general cellular changes or changes in luciferase turnover. The 5-fold difference in promoter activity responsive to DEX between minigenes containing 140 bp versus

Figure 6 Linker scanning analysis of the putative BCKAD-E2 GRU

A series of linker scanning mutations were introduced in the 5' upstream base sequence of the E2 promoter region to create novel *SacI* sites at the positions indicated (Figure 5). Each minigene was transfected into H4IIEC3 cells ($n = 3$) and alkaline phosphatase and luciferase activities determined. The relative increases in the luciferase activities standardized for individual transfection efficiencies (LUC/SEAP) observed in the presence of DEX (mean value $\pm$ S.D.) are based on the basal activities of each of the minigenes tested in the absence of DEX being assigned a value of 100% for the comparison. Only two or more -fold differences (200% or 50%) from control were statistically significant ($P < 0.05$).

76 bp of 5' upstream region suggested that this upstream region might contain a GRU [31].

The minimum BCKAD promoter sequence that could be demonstrated contained 44 bases upstream from the transcription start site (Figure 3) [27]. Further deletions demonstrated no statistically significant luciferase activities above that produced by promoterless minigenes. Similarly, none of these smaller minigenes demonstrated significant changes in the presence of DEX or DEX and dBcAMP (results not shown). A full sequence map of the murine BCKAD E2 genomic region affecting glucocorticoid responsiveness and minimal promoter activity (–140 bp through exon 1 sequence) with potential transcription-factor-binding sites (based on sequence analysis) is shown in Figure 4.

Characterization of the BCKAD-E2 GRU

The region –140 to –70 bp upstream from the start site was examined by constructing a series of 7 minigenes containing base substitutions scattered throughout the region (linker scanning series pGR1–pGR7). These base substitutions were designed to introduce internal *SacI* sites, located at sites progressively closer to the transcription start site, so that a corresponding series of minigenes containing progressively larger deletions in this region could be constructed as well (pGRL1–7). These are outlined in Figure 5 along with their assessed basal promoter activities when transfected into H4IIEC3 cells.

The linker scanning series pGR1–pGR7 were transiently transfected into H4IIEC3 cells and their basal activities compared to the activity obtained with the parent wild-type construct, pGL53, which contained the unaltered BCKAD promoter sequence from –140 to +8 bp (Figure 5). The basal promoter activity of the altered sequences was shown to decrease in some, but increase in other, mutated plasmids (Figure 5, top). For example, muta-

Figure 7 Deletion analysis of the putative BCKAD-E2 GRU

The series of linker scanning mutations introduced in the 5' upstream base sequence of the E2 promoter region to create novel *SacI* sites were used to create a series of minigenes with increasing sized deletions of the region between 140 and 76 bases upstream from the transcription start site (Figure 5). Each minigene was transfected into H4IIEC3 cells ($n = 3$) and alkaline phosphatase and luciferase activities determined. The relative increases in the luciferase activities standardized for individual transfection efficiencies (LUC/SEAP) observed in the presence of DEX (mean value $\pm$ S.D.) are based on the basal activities of each of the minigenes tested in the absence of DEX being assigned a value of 100% for the comparison. Only two or more -fold differences (200% or 50%) from control were statistically significant ($P < 0.05$).

genesis in the reverse CAAT box area (pGR7) resulted in a 60–70% decrease in the basal promoter activity of the sequence. Similar (40–60%) decreases in basal activity were observed when pGR2 and pGR6 were used, both of which contained changes in polypyrimidine regions. The pGR5 minigene (substitution at –84 to –92) demonstrated slightly increased basal activity (40–50%). When tested in the presence of DEX, the associated increase in activities of pGR1, pGR2, pGR3, pGR4, and pGR5 were significantly lower compared to that observed using the unaltered pGL53, showing 3-fold versus 6-fold elevations in activities (Figure 6). In contrast, both pGR6 and pGR7 (altered CAAT site) demonstrated no change in responsiveness to DEX (6-fold increases) compared to unaltered pGL53 despite lowered basal activity (Figure 5 for basal activity, Figure 6 for DEX responsiveness).

Analysis of the promoter activity of the minigenes containing the progressively deleted sequences from –140 to –101 bp (pGRL1–4) demonstrated no significant changes in basal activities, but further deletion to –92 (pGRL5), –85 (pGRL6) and –76 (pGRL7) bp demonstrated significantly decreased promoter activities (Figure 5, bottom). There appeared to be an approx. 10-fold decrease in promoter activity with the loss of the sequences from –140 to –76 bp. In contrast to this trend of decrease in basal activities observed associated with increasing deletions towards the 3' area of this region, the effects on the apparent DEX responsiveness were significantly different from unaltered GL53 in only 3 of the plasmids: one in the 5' region (deletion of –140 to –127, 2-fold versus 5-fold increases with DEX) and two in the 3' region (to –85, 2-fold increase with DEX; to –76, 0.5-fold increase with DEX) (Figure 7).

Based on the above mutagenesis and deletion studies, there appeared to be two different areas responsible for DEX re-

Figure 8 Combinatorial effects of linker scanning mutations in the BCKAD E2 GRU region

BCKAD E2 minigenes containing linker-scanning mutations at -127 (GR1), -116 (GR2) and -76 (GR7) individually or in combination (GR71, at both -127 and -76 , and GR72, at both -116 and -76) were transfected into H4IIEC3 cells ($n = 3$) and SEAP and luciferase activities measured prior to and after the addition of dexamethasone (DEX, $1.0 \mu\text{M}$). Relative promoter activities are indicated as the % increase in luciferase (LUC) activity (standardized to SEAP) observed following DEX addition, compared to control transfected cells, for each of the individual minigenes.

sponsiveness in the putative GRU region. To determine if mutagenesis of both sites abolished DEX responsiveness, two more mutant minigenes (pGR71 and pGR72) were constructed containing mutations at both the 3' CAAT site (GR7, -76 to -85) and the 5' SDR site (GR1, -127 to -134) or the contiguous 5' polypyrimidine site (GR2, -116 to -127). The combination of two mutated sites in each of the two minigenes created resulted in a significant decrease in the basal, uninduced activity of the promoter (Figure 5). The effect of DEX on cells transfected with these two minigenes was compared to that containing the three individual mutations or the unaltered sequence (pGL53) (Figure 8). Introduction of the GR1 (-127 to -134) mutation in combination with the GR7 (-76 to -85) mutation significantly impaired the increase in promoter activity associated with DEX in this minigene (pGR71). The introduction of the GR2 (-116 to -127) mutation in combination with GR7 not only impaired, but completely abolished, the increases otherwise observed in the presence of DEX (pGR72). These findings demonstrated that there may be at least two *cis*-acting regulatory elements within the GRU region which may in combination affect the glucocorticoid-associated increase in E2 promoter activity.

Protein–DNA interaction in the BCKAD GRU region

To determine if the BCKAD GRU region contained sites for specific DNA–protein complexes, gel-shift analysis was performed using oligonucleotide sequence containing the -47 to -140 bp region upstream from the initiation start site (Figure 9; P indicates labelled oligonucleotide). Using nuclear extracts prepared from H4IIEC3 cells, the formation of several shifted bands was identified, indicating binding of nuclear proteins to this region of promoter sequence (Figure 9, lane 2). All of the shifted bands were efficiently competed with excess unlabelled E2

Figure 9 Gel-retardation analysis of the BCKAD upstream promoter region

Oligonucleotide DNA prepared from the murine BCKAD E2 promoter region (-47 to -140 bp upstream from the transcription initiation site) was prepared as a probe, labelled with $[\gamma\text{-}^{32}\text{P}]$, incubated with nuclear extract from H4IIEC3 cells and analysed by non-denaturing PAGE as described in the Experimental section. Competitor DNA was prepared from unaltered wild-type murine sequence pGL53 (lanes 3 top and bottom) and from the series of altered sequences containing linker scanning mutations (pGR1–7, lanes 4–10 top), double mutation containing (pGR71 and 72, lanes 11–12 top) or contiguously deleted sequences (pGRL 1–7, bottom lanes 4–10). P indicates the labelled oligonucleotide free of nuclear protein binding, as indicated in lane 1 in both top and bottom series. The arrowhead marks the oligonucleotide's retarded migration due to nuclear protein binding in the absence of competitor DNA (lane 2, top and bottom). The sequences contained in all competitor DNA can be viewed in Figure 5.

oligonucleotide sequence, but not with oligonucleotides containing SP1 or AP2 consensus sequences (results not shown). The top, slower migrating band (arrow) was easily competed with as little as $100\times$ excess unlabelled probe, but the lower, more quickly migrating bands required at least $200\times$ excess of competitor. Using the same sequences (-47 to -140) prepared from pGL53, the wild-type sequence (lane 3), or the series of linker scanning mutation containing minigenes pGR1–7 (top lanes 4–10), pGR71 and pGR72 (top lanes 11–12) and the contiguous deletion series pGRL1–7 (bottom lanes 4–10), the ability to

compete out these bands was analysed. The upper band (arrowhead) was competed out with all the mutant competitors as efficiently as the wild-type sequence, with the exception of competitor DNA with mutations in or near the reverse CAAT box site at -78 to -83 (pGR6, pGR7, pGR71, pGR72, upper lanes 9–12). Similarly, the competitors containing sequences extending upstream from the CAAT box site (pGRL1–pGRL5, lower lanes 4–8) competed the shifted band efficiently. However, those sequences prepared from the minigene pGRL7 (lower lane 10), containing only sequences -47 to -76 , and thereby lacking the CAAT site, failed to out compete the shifted band. The pGRL6 derived oligonucleotide (-47 to -85) which contained sequences directly upstream from the CAAT site deleted, was less efficient in competing the shifted band (lower lane 9). Similar ineffective competition was observed when the pGR6 minigene-derived competitor (mutation just proximal to the CAAT site) was used (upper lane 9). Nuclear extracts prepared from HeLa cells demonstrated similar results to that observed with the H4IIEC3 cells, except that the only mutated DNA that could not compete the upper shifted band was again DNA derived from pGR7 (results not shown). These data suggested that DNA–protein interactions in the CAAT-box area of the E2 GRU region likely occur but are not hepatocyte or rodent specific.

DISCUSSION

In response to developmental, dietary and hormonal changes, alterations in hepatic BCKAD E2 RNA levels may be observed, with increases or decreases of 2–4 fold from pretreatment or control steady-state levels. Because of the known associated changes in circulating glucocorticoid levels associated with some of these conditions, it was suggested that regulation of E2 gene expression may be influenced by these hormones. The results in this paper of the influence of glucocorticoids on BCKAD E2 promoter activity in hepatoma cell line H4IIEC3 supports the mechanism for regulation of BCKAD E2 gene expression. We have demonstrated that a genomic region located -140 to -70 bp upstream from the transcription initiation site appears to function as a GRU. Promoter activity of the E2 gene is likely subject to the interplay of multiple factors interacting with a number of promoter proximal and distal sequences. However, this region appears to play a major role in ensuring that a 5–10 fold increase from the lowest basal levels of promoter activity can be produced, and this effect may be in large part influenced by glucocorticoids. The exact mechanism by which this comes about remains to be determined. Our results suggests that there may be an interplay between processes occurring at different specific oligonucleotide sequences in this GRU region, which individually may not demonstrate a full noticeable effect, but in concert produce very evident changes.

Using nuclear extracts from H4IIEC3 cells, preliminary gel-mobility-shift experiments have now demonstrated that protein binding in the region of -140 to -47 bp upstream from the initiation site is affected by mutations in or near the reverse CAAT box site at -78 to -83 (ATTGGC). Highly detailed kinetic-binding studies will be required to establish the dynamics of DNA–protein interaction in this region. These preliminary studies nonetheless support the proposal that this GRU region plays a central role in the response of the E2 promoter region to external stimuli, such as glucocorticoids.

Further evidence for an important regulatory role of this region is revealed by sequence comparison of murine and human E2 genomic sequences. The proximal 140 bp of the human E2 promoter is similar in sequence to that of the murine E2 promoter [27,32]. Sequence identity is especially high in the region between

-69 to -140 (80%), compared to 75% sequence identity observed throughout the exon 1 region ($+1$ to $+64$ bp; encodes 95% amino acid identity). This high level of identity suggested that *cis*-acting elements that are important for the regulation of the E2 gene might be located in this region. Preliminary gel-shift experiments with human oligonucleotide sequence similarly competes with the apparent protein–DNA interaction occurring in this murine E2 region of -83 to -77 (results not shown). Further evidence will have to await studies of human E2 promoter interactions with glucocorticoids.

The association of variable basal activities with linker-scanning type mutations in this 70 bp GRU region, suggests composite multiple transcription factors may play a role in regulating overall E2 promoter activity at any given time, similar to that observed in other hepatic genes responsive to hormonal stimuli (e.g. phosphoenolpyruvate carboxykinase, [31]). The fact that there was no correlation between loss of relative DEX effectiveness and loss of otherwise basal promoter activity (e.g. GR7 has the lowest basal activity but retained similar DEX responsiveness to that observed with the non-mutated minigene, see Figure 6), suggests that regulation of E2 promoter activity through glucocorticoid interaction provides a major influence on expression of BCKAD. Other BCKAD subunits have been observed to be affected by glucocorticoids. Under some experimental conditions, both E1 α and E2 RNAs have been observed to increase in the presence of glucocorticoids in hepatic and non-hepatic cells [21,22,33]. Recent studies have demonstrated that a major effect of glucocorticoids in hepatic cells may be to down-regulate the amount of BCKAD kinase responsible for phosphorylating, and thus inactivating, the BCKAD E1 α subunit [21]. Although steady-state levels of kinase RNA appear to be affected in the presence of glucocorticoids, no direct effect of glucocorticoids on promoter activity of the kinase gene could be demonstrated with the minigene kinase sequences available [21]. Nonetheless, the combination of providing a mechanism of ensuring increased levels of active, non-phosphorylated, E1 α subunit (by decreasing levels of BCKAD kinase) to associate with increased levels of E2 subunit, would give glucocorticoids a central role in regulation of overall BCKAD complex activity by affecting expression at multiple levels. These studies support the concept that the regulation of cellular BCKAD activity may involve the availability of subunits for association in addition to phosphorylation [20,34]. The studies presented in this report demonstrate that the availability of the E2 subunit, upon which the other BCKAD subunits assemble, may be regulated at the level of transcription by glucocorticoid influence on E2 promoter activity. The direct role of glucocorticoids in the regulation of the expression of the genes encoding the other BCKAD subunits remains to be determined.

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