Chicken ovalbumin upstream-promoter transcription factor and E-box-binding proteins enhance thyroid-hormone responsiveness of the malic enzyme gene in avian hepatocytes

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In chick embryo hepatocytes (CEH), stimulation of malic enzyme transcription by 3,3',5-tri-iodothyronine (T3) is mediated by a liver-specific and T3-inducible DNase I hypersensitive region (–3910 to –3640 bp) in the malic enzyme gene. Previous studies have shown that this region contains a cluster of five T3 response elements (T3REs), referred to as a T3 response unit (T3RU), plus three accessory elements that enhance T3 responsiveness conferred by the T3RU. Here we report the identification of two additional accessory elements within the –3910 to –3640 bp region. Each element augments T3 regulation of malic enzyme transcription in CEH. One element, designated region G (–3681/–3666 bp), contains a single nuclear-hormone-receptor half-site that binds the orphan receptor chicken ovalbumin upstream-promoter transcription factor. The other element, designated region H (–3655/–3646 bp), contains an E-box motif that binds proteins of unknown identity. Stimulation of T3RE function by region G or region H does not require the presence of additional malic enzyme sequences. In contrast with

the stimulatory effects of regions G and H on T3 responsiveness in CEH, neither of these elements is effective in modulating T3 responsiveness in chick embryo fibroblasts (CEF). Instead, region H functions as a T3-insensitive repressor of transcription in CEF. These results indicate that chicken ovalbumin upstream-promoter transcription factor and E-box-binding proteins interact with nuclear T3 receptors to enhance T3 regulation of malic enzyme transcription in CEH and that alterations in region G and region H activities contribute to diminished T3 regulation of malic enzyme transcription in CEF relative to CEH. As the pattern of protein binding to regions G and H varies substantially between CEH and CEF, the mechanism for cell-type-dependent differences in region G and region H activity may involve alterations in protein binding to these T3 accessory elements.

Key words: enhanceosome, fatty acid synthesis, lipogenesis, nuclear T3 receptor, orphan receptor.

INTRODUCTION

Malic enzyme is a member of a group of adaptive enzymes involved in the *de novo* synthesis of long-chain fatty acids. Malic enzyme catalyses the oxidative decarboxylation of malate to pyruvate and CO₂, simultaneously generating NADPH from NADP⁺. In avian liver, this reaction provides most of the NADPH required for fatty acid synthesis [1]. Malic enzyme activity is regulated by changes in nutritional status. For example, feeding previously starved chicks a high-carbohydrate, low-fat diet stimulates a 70-fold increase in malic enzyme activity in liver, and this effect is reversed when animals are subsequently starved [2]. Several lines of evidence indicate that thyroid hormone plays an important role in mediating changes in malic enzyme activity caused by nutritional manipulation. First, the stimulation of malic enzyme activity caused by feeding previously starved animals a high-carbohydrate, low-fat diet is preceded by increases in the levels of the active form of thyroid hormone, 3,3',5-tri-iodothyronine (T3), in the blood [3]. Secondly, hypothyroidism in high-carbohydrate-fed animals causes a marked decrease in malic enzyme activity in liver [4]. Thirdly, T3 stimulates a 40-fold

increase in malic enzyme activity in primary cultures of chick embryo hepatocytes (CEH) [5]. In addition to the direct effects of T3 on malic enzyme activity, this hormone potentiates the actions of other humoral factors on malic enzyme activity in CEH. For example, regulation of malic enzyme activity by insulin, corticosterone, glucose, glucagon and fatty acids requires the presence of T3 in the culture medium [5–8]. Alterations in malic enzyme activity caused by nutritional manipulation *in vivo* and nutrients and hormones in CEH are mediated primarily by changes in the rate of transcription of the malic enzyme gene [6,9,10].

In contrast with liver, alterations in nutritional status have no effect on malic enzyme transcription in heart, kidney and brain of intact chicks [9]. Regulation of malic enzyme transcription by T3 is also dramatically diminished in non-hepatic cell lines, such as chick embryo fibroblasts (CEF) and quail QT6 cells [11]. Decreased T3 responsiveness in CEF relative to CEH is due to alterations in malic enzyme transcription in both the absence and presence of T3. In the absence of T3, malic enzyme transcription in CEF is 6-fold higher than that observed in CEH, whereas in the presence of T3, malic enzyme transcription in CEH is about

Abbreviations used: CEH, chick embryo hepatocytes; CEF, chick embryo fibroblasts; CAT, chloramphenicol acetyltransferase; T3, 3,3',5-tri-iodothyronine; TR, nuclear T3 receptor; COUP-TF, chicken ovalbumin upstream-promoter transcription factor; RXR, retinoid X receptor; RAR, retinoic acid receptor; T3RE, T3 response element; T3RU, T3 response unit; DR4, directly repeated hexameric half-sites separated by a 4 bp spacer; USF, upstream stimulatory factor; PEPCCK, phosphoenolpyruvate carboxykinase; CREB, cAMP-response-element-binding protein; SREBP, sterol regulatory element-binding protein.

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Figure 1 Schematic representation of the DNase I-hypersensitive region between -3910 and -3640 bp

A T3-inducible and liver-specific DNase I-hypersensitive region between -3910 and -3640 bp mediates most of the T3 regulation of malic enzyme transcription in CEH. Within this region is a cluster of five T3REs (T3RE-2 to T3RE-6) that bind TR-RXR heterodimers. This region is referred to as the T3RU. Flanking the T3RU are additional protein-binding sites detected in DNase I-footprinting assays. These sites are regions A, E, F, G and H. Previous studies have shown that regions A, E and F enhance T3 responsiveness conferred by the T3RU [19,20]. Region E binds protein complexes containing the homeodomain proteins PBX and MEIS1. Region F binds the liver-enriched protein CCAAT/enhancer-binding protein (C/EBP). The proteins that bind region A have not yet been identified. The effects of regions G and H on T3 action and the proteins that bind these DNA elements were investigated in the present study.

6-fold higher than that observed in CEF [11]. Thus, in addition to nutrients and hormones, the malic enzyme gene is controlled by cell- or tissue-specific factors.

T3 activates gene transcription by interacting with nuclear T3 receptors (TRs) [12]. TRs bind T3 response elements (T3REs) of target genes as homodimers or heterodimers with the retinoid X receptor (RXR). The T3-induced increase in malic enzyme transcription in CEH is mediated by at least six T3REs [13–15]. One strongly active and four weakly active T3REs are located at $-3878/-3769$ bp relative to the transcription start site. This cluster of T3REs is referred to as a T3 response unit (T3RU; Figure 1). Another weakly active T3RE is located about 700 bp downstream of the T3RU. All of the malic enzyme T3REs consist of directly repeated hexameric half-sites separated by a 4 bp spacer (DR4) and bind TR-RXR heterodimers in hepatic nuclear extracts. Decreased T3 regulation of malic enzyme transcription in CEF relative to CEH is mediated in part by a reduction in the binding of TR-RXR heterodimers to the strongly active T3RE (T3RE-2) in the malic enzyme T3RU [15]. An alteration in RXR α expression accounts for the difference in TR-RXR binding activity between CEH and CEF.

Each of the T3REs contained in the malic enzyme T3RU confers T3 responsiveness by inhibiting transcription in the absence of T3 and activating transcription in its presence [13,14]. This observation is consistent with previous data demonstrating that the unliganded TR functions as a repressor of transcription and that binding of T3 to TR reverses this effect and, in some instances, stimulates transcription above that observed in the absence of TR [11,16]. The dual regulatory activity of TR arises, in part, from the ability of TR to recruit co-repressor and co-activator complexes that lack intrinsic DNA-binding activity. Several of the proteins contained in these complexes have been identified and include nuclear receptor co-repressor, silencing mediator for retinoic acid receptor (RAR) and thyroid hormone receptor, steroid receptor co-activator-1, glucocorticoid receptor-interacting protein 1, thyroid hormone receptor-associated protein 220 and cAMP-response-element-binding protein (CREB)-binding protein [16–18].

In addition to co-repressor and co-activator complexes, accessory transcription factors that bind *cis*-acting elements distinct from T3REs play an important role in modulating T3 action. We have identified four accessory elements in the malic enzyme gene that confer differences in T3 responsiveness between CEH and CEF [19]. Each element enhances T3 responsiveness in CEH but has no effect on T3 responsiveness in CEF. Three of the accessory elements immediately flank the T3RU and are designated regions A ($-3895/-3890$ bp), E ($-3761/-3744$ bp) and F ($-3703/-3686$ bp; Figure 1). Region F binds the liver-enriched factor, CCAAT/enhancer-binding protein [19], and region E binds complexes containing PBX and MEIS1 [20], members of the TALE (three-amino-acid loop extension) class of homeodomain proteins. Stimulation of T3 responsiveness by region E is mediated, at least in part, by direct interactions between PBX-MEIS1 complexes and TR-RXR heterodimers [20].

In hepatocytes, the T3RU and accessory elements A, E and F overlap a hepatocyte-specific and T3-inducible region of DNase I hypersensitivity (-3910 to -3640 bp) in chromatin [19]. The 3' boundary of this DNase I-hypersensitive region extends about 50 bp downstream of region F, suggesting the presence of additional T3 regulatory sequences 3' of this accessory element. Here we report the identification of two additional TR accessory elements flanking the T3RU in the malic enzyme gene. Both of these elements augment T3 responsiveness in CEH but have no effect on T3 responsiveness in CEF. One element, designated region G ($-3681/-3666$ bp), contains a single nuclear-hormone-receptor half-site that binds the orphan receptor, chicken ovalbumin upstream-promoter transcription factor (COUP-TF). The second element, designated region H ($-3655/-3646$ bp) contains an E-box motif that binds protein complexes of unknown identity.

MATERIALS AND METHODS

Plasmids

The DNA fragments used to construct reporter plasmids were named by designating the 5' and 3' ends of each fragment relative

to the start site of transcription of the malic enzyme gene. p[ME-3921/-3631]ME-147/+31CAT has been described previously [19]. This reporter plasmid contains the malic enzyme T3RU and flanking sequences (–3921 to –3631 bp) linked to the minimal promoter of the malic enzyme gene (–147 to +31 bp). Site-directed mutations were introduced into p[ME-3921/-3631]ME-147/+31CAT using a PCR-based strategy [21]. p[T3RE-2]ME-147/+31CAT was made by subcloning the major malic enzyme T3RE (–3883 to –3858 bp) into the *Bam*HI/*Sac*I site upstream of the minimal promoter in ME-147/+31CAT. Region G (ME-3681/-3666) and region H (ME-3655/-3646) were inserted into the *Spe*I/*Sma*I site immediately downstream of the T3RE-2 in p[T3RE-2]ME-147/+31CAT to form p[T3RE-2+G]ME-147/+31CAT and p[T3RE-2+H]ME-147/+31CAT, respectively. Dr M.-J. Tsai (Baylor College of Medicine, Houston, TX, U.S.A.) provided the cDNAs for human COUP-TFI and COUP-TFII (also known as ARPI). The cDNA for human RXR α was provided by Dr R. Evans (Salk Institute, La Jolla, CA, U.S.A.). cDNAs for COUP-TFI, COUP-TFII and RXR α were subcloned into pSV-SPORT1 (Gibco BRL). Structures of reporter plasmids and expression plasmids were confirmed by restriction-enzyme mapping and nucleotide sequence analyses.

Cell culture and transient transfection

Primary cultures of CEH were prepared as described previously [22] and maintained in serum-free Waymouth medium MD705/1 containing 50 nM insulin (a gift from Eli Lilly Corp.) and 1 μ M corticosterone. Hepatocytes were incubated at 40 °C in a humidified atmosphere of 5% CO₂/95% air. Cells were transfected using a modification of the method of Baillie et al. [23]. Briefly, CEH were isolated as described above and plated on to 35 mm dishes. After 18 h of incubation, the medium was replaced with one containing 20 μ g of LipofectACE (Gibco BRL), 3.0 μ g of p[ME-3921/-3631]ME-147/+31CAT or an equimolar amount of another reporter plasmid and 0.05 μ g of pCMV- β -galactosidase (internal transfection standard). After 42 h of incubation, the transfection medium was replaced with fresh medium with or without T3 (1.5 μ M). After 90 h of incubation, CEH were harvested and cell extracts were prepared for chloramphenicol acetyltransferase (CAT) [24] and β -galactosidase [25] measurements. CAT activity was expressed relative to β -galactosidase activity to correct for differences in transfection efficiency between samples. All DNAs used in transfection experiments were purified using the Qiagen endotoxin-free kit.

CEF were obtained from SPAFAS (Norwich, CT, U.S.A.) and were cultured routinely in Dulbecco's modified Eagle's medium (containing 25 mM glucose) and Medium 199 in a 1:1 ratio (v/v), containing 10000 units/l penicillin G, 10 mg/l streptomycin sulphate and 25 μ g/l amphotericin B (DMEM/M199) supplemented with 5% fetal bovine serum. CEF were transfected with supercoiled DNAs using the calcium phosphate method [26]. Briefly, CEF were seeded on T-75 flasks and grown in DMEM/M199 containing 5% fetal bovine serum until the cells were 70% confluent. Before transfection (24 h), medium was changed to DMEM/M199 containing 5% fetal bovine serum depleted of thyroid hormone [27]. The cells were incubated in this medium throughout the experiment. Cells were transfected with 15 μ g of p[ME-3921/-3631]ME-147/+31CAT or an equimolar amount of another reporter plasmid and 0.7 μ g of pCMV- β -galactosidase. Exposure to the calcium phosphate/DNA precipitate was for 16 h. The transfected cells were trypsinized and distributed to 60 mm tissue-culture plates. T3 was added to the medium at this time. After 48 h of incubation,

cells were harvested and extracts were prepared for CAT and β -galactosidase assays.

Gel mobility-shift analysis

Nuclear extracts were prepared from CEH and CEF incubated with or without T3 for 24 h. Nuclei and nuclear extracts were prepared as described in [28] except that the protease inhibitors, leupeptin (0.25 μ g/ml), benzamide (10 mM) and PMSF (0.5 mM), were added to the extraction and dialysis buffers at the indicated concentrations. COUP-TFI, COUP-TFII and RXR α were translated *in vitro* using the TNT SP6-coupled reticulocyte lysate system (Promega). Incorporation of [³⁵S]methionine into COUP-TFI, COUP-TFII and RXR α was measured in parallel reactions to assess the relative efficiency of synthesis of the different transcription factors.

Double-stranded oligonucleotides were prepared by combining equal amounts of the complementary single-stranded DNA in a solution containing 10 mM Tris, pH 8.0, and 1 mM EDTA followed by heating to 95 °C for 2 min, and then cooling to room temperature. The annealed oligonucleotides were labelled by filling in overhanging 5' ends using the Klenow fragment of *Escherichia coli* DNA polymerase in the presence of [α -³²P]dCTP. Binding reactions were carried out in 20 μ l containing 18 mM Hepes, pH 7.9, 90 mM KCl, 0.18 mM EDTA, 0.45 mM dithiothreitol, 18% glycerol (v/v), 0.3 mg/ml BSA and 2 μ g of poly(dI-dC). A typical reaction contained 20000 c.p.m. of labelled DNA and 10 μ g of nuclear extract or 2 μ g of *in vitro*-translated proteins. The reaction was carried out on ice for 60 min. DNA and DNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels at 4 °C in 0.5 $\times$ TBE (where 1 $\times$ TBE is 45 mM Tris, pH 8.3/45 mM boric acid/1 mM EDTA). Following electrophoresis, the gels were dried and the radioactivity in the gels visualized using a PhosphorImager.

For competition experiments, unlabelled competitor DNA was mixed with radiolabelled oligomer prior to addition of nuclear extract. For gel supershift experiments, nuclear extracts or *in vitro*-translated proteins were incubated with antibodies for 1 h at 0 °C prior to addition of the oligonucleotide probe. An antiserum reacting with COUP-TFI and COUP-TFII was provided generously by Dr M.-J. Tsai. Dr P. Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Strasbourg, France) kindly provided a monoclonal antibody that recognizes the α , β and γ forms of RXR. An antibody against the α and β forms of TR (FL-408) was obtained from Santa Cruz Biotechnology. Synthetic oligonucleotides that were used as probes or competitors in gel mobility-shift assays are listed in Figures 4 and 7 (see below).

RESULTS

Malic enzyme regions G and H enhance T3 regulation of transcription in CEH but not in CEF

Previous results from DNase I-hypersensitivity and DNase I-footprinting analyses suggested the presence of protein-binding sites immediately 3' of region F of the malic enzyme gene [19]. These sequences are located between –3681 and –3666 bp and between –3655 and –3646 bp relative to the transcription start site, and are designated region G and region H, respectively (Figure 1). To determine whether region G and region H modulate T3 responsiveness, transient-transfection experiments were performed with reporter constructs containing block mutations in region G (mut G block) or region H (mut H block) in the context of p[ME-3921/-3631]ME-147/+31CAT. Mut G block and mut H block decreased T3 responsiveness by 42 and

Figure 2 Mutations of region G and region H of the malic enzyme gene inhibit T3 regulation of transcription in CEH but not in CEF

p[ME-3921/-3631]ME-147/+31CAT or constructs containing block mutations of region G (mut G block) or region H (mut H block) in the context of p[ME-3921/-3631]ME-147/+31CAT were transiently transfected into CEH and CEF as described in the Materials and methods section. All transfections contained CMV- β -galactosidase as a control for transfection efficiency. After the transfection, cells were treated with or without T3 for 48 h. Cells were then harvested, extracts prepared, and CAT and β -galactosidase assays performed. In (B) and (C), the left-hand panels indicate the constructs used in these experiments. Numbers indicate the 5' or 3' boundaries of malic enzyme DNA in nucleotides relative to the start site for transcription. In (B) and (C) right-hand panels, CAT activity was expressed relative to β -galactosidase activity to correct for differences in transfection efficiency. CAT activity in cells transfected with p[ME-3921/-3631]ME-147/+31CAT and incubated without T3 was set at 1, and the other activities were adjusted proportionately. The fold stimulation by T3 was calculated by dividing CAT activity in hepatocytes treated with T3 (+T3) by that for hepatocytes not treated with T3 (-T3). The fold responses were calculated for individual experiments and then averaged. The results are the means $\pm$ S.E.M. from five experiments. (A) Sequences of wild-type and mutant forms of regions G and H. (B) Results of experiments performed with CEH. (C) Results of experiments performed with CEF. Asterisks indicate that the fold stimulation by T3 was significantly lower than that of cells transfected with p[ME-3921/-3631]ME-147/+31CAT ($P < 0.05$).

50 %, respectively, in CEH (Figure 2). For CEH, the effect of mut G block on T3 responsiveness was due to an increase in promoter activity in the absence of T3 and a decrease in promoter activity in the presence of T3, whereas the effect of mut H block was due

only to an increase in promoter activity in the absence of T3. In contrast with these data for CEH, mut G block and mut H block had no effect on T3 responsiveness in CEF. Together, these results suggest that regions G and H play a role in mediating cell-

Figure 3 Characterization of proteins that bind to region G

Gel mobility-shift experiments were performed using nuclear extracts prepared from CEH and CEF. A double-stranded DNA fragment containing region G (–3682 to –3662 bp) was labelled with [α - 32 P]dCTP using the Klenow fragment of *E. coli* DNA polymerase. The radiolabelled probe was incubated with 10 μ g of nuclear protein in the absence and presence of antibody as described in the Materials and methods section. DNA and DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels. Positions of specific protein–DNA complexes (arrows), supershifted complexes (bracket) and non-specific complexes (asterisk) are indicated. These data are representative of four different experiments employing independent preparations of nuclear extract.

type-dependent differences in T3 regulation of malic enzyme transcription. Although mut H block had no effect on T3 responsiveness in CEF, this mutation stimulated a marked increase (greater than 7-fold) in promoter activity in these cells in both the absence and presence of T3. Thus region H functions as a T3-insensitive repressor of transcription in CEF.

Characterization of proteins that bind region G

Gel mobility-shift analyses were performed to characterize the proteins that interacted with region G. Incubation of a labelled oligonucleotide probe containing region G (–3682 to –3662 bp, wt G) with nuclear extracts from CEH and CEF resulted in the formation of three protein–DNA complexes that were designated 1, 2 and 3 in the order of increasing mobility (Figure 3). The abundance of complex 3 in CEH was substantially higher than that of complexes 1 and 2, whereas in CEF complexes 1 and 2 were markedly more abundant than complex 3. T3 treatment did not affect the pattern or intensity of protein binding to region G in CEH and CEF (results not shown). The presence of a 50-fold molar excess of unlabelled oligonucleotide probe abolished the formation of complexes 1–3, indicating that the binding of these protein–DNA complexes was specific (Figure 4).

Region G contains a sequence at –3678/–3673 bp (5'-AGGTCA-3') that perfectly matches the consensus half-site sequence for nuclear hormone receptors. Previous studies have demonstrated that COUP-TFI and the highly related factor ARPI can bind single nuclear-hormone-receptor half-sites in liver cells [29,30]. For simplicity we will refer to COUP-TFI and

Figure 4 A single nuclear-hormone-receptor half-site is required for protein binding to region G

Gel mobility-shift experiments were performed as described in the Materials and methods section using a 32 P-labelled DNA probe (–3682 to –3662 bp) containing region G and CEH nuclear extract (lanes 1–6), *in vitro*-synthesized COUP-TFI (lanes 7–12) or CEF nuclear extract (lanes 13–18). Incubations with the region G probe were performed in the absence and presence of unlabelled competitor DNAs (50-fold molar excess). DNA and DNA–protein complexes were resolved on 6% non-denaturing polyacrylamide gels. (A) The sequences of the competitor DNAs. wt G is the native malic enzyme sequence between –3682 and –3662 bp. The nuclear-hormone-receptor half-site contained in wt G is indicated by the boxed sequence in bold type. mut G1, mut G2, mut G3 and mut G4 contain 3 bp mutations within the –3682 to –3662 bp sequence. (B) Competition analysis with native and mutant competitor DNAs. These data are representative of four experiments employing independent preparations of nuclear extract.

ARPI as COUP-TF. Antibody-supershift experiments were performed to determine whether COUP-TF bound region G. Preincubation of nuclear extracts from CEH or CEF with antibodies against COUP-TF disrupted the formation of complexes 1–3 (Figure 3). However, antibodies against the α , β and γ isoforms of RXR, proteins that are capable of forming heterodimers with COUP-TF [31], had no effect on the formation of complexes 1–3. Antibodies against TR also had no effect on protein binding to region G (results not shown). The disruption of complexes 1–3 by COUP-TF antibodies was associated with the appearance of supershifted complexes. These results indicate that complexes 1–3 contain COUP-TF but not RXR or TR. The mobilities of complexes 1–3 were comparable with or lower than that of a protein–DNA complex containing *in vitro*-synthesized TR α and RXR α bound to a T3RE probe of similar size to the region G probe (results not shown). This observation suggested that COUP-TF in complexes 1–3 was in the form of a dimer or higher-order complex.

To confirm that COUP-TF bound region G, gel mobility-shift experiments were performed using *in vitro*-synthesized proteins.

Figure 5 Region G binds homodimers containing COUP-TFI or ARP1

Gel mobility-shift assays were performed as described in the Materials and methods section using a 32 P-labelled DNA probe (–3682 to –3662 bp) containing region G. In lanes 5–16, equimolar amounts of *in vitro*-synthesized COUP-TFI or ARP1 were incubated with radiolabelled probe in the absence or presence of *in vitro*-synthesized RXR as indicated. In lanes 6, 8, 10, 12, 13, 15 and 16, *in vitro*-synthesized proteins were incubated with antibodies against COUP-TF or RXR prior to the addition of the probe. In lanes 2 and 3, the radiolabelled probe was incubated with nuclear extract from CEH and CEF, respectively. Positions of complexes 1–3 (lanes 2 and 3) and COUP-TFI and ARP1 homodimeric complexes (lanes 5–16) are indicated by arrows.

Incubation of the region G probe with *in vitro*-synthesized COUP-TFI or ARP1 resulted in the formation of a specific complex whose mobility was similar to that of complex 1 in nuclear extracts of CEH and CEF (Figure 5). This complex was disrupted by the presence of antibodies against COUP-TF. *In vitro*-synthesized RXR α alone or in combination with COUP-TFI or ARP1 had no effect on the pattern of protein binding to region G. These results demonstrate further that region G binds COUP-TF-COUP-TF dimers but not COUP-TF-RXR heterodimers.

To identify the sequences in region G that mediated the binding of COUP-TF complexes, competition experiments were performed with unlabelled oligonucleotides containing 3 bp mutations spanning the –3682/–3662 bp region. Oligonucleotides containing mutations that flanked the half-site motif [e.g. mut G1 (–3681/–3679 bp), mut G3 (–3672/–3670 bp) and mut G4 (–3667/–3665)] were effective in competing for the binding of complexes 1–3 in CEH and CEF, whereas an oligonucleotide containing a mutation within the half-site motif [e.g. mut G2 (–3677/–3675)] was not effective in competing for the binding of complexes 1–3 (Figure 4). A similar competition profile was observed for *in vitro*-synthesized COUP-TFI bound to region G. These data indicate that the binding of COUP-TF to region G requires the presence of the half-site at –3678/–3673 bp and that sequences flanking this motif have little or no effect on binding activity.

To investigate whether COUP-TF binding to region G was required for optimal T3 responsiveness in CEH, transfection analyses were performed with reporter constructs containing site-specific mutations of region G in the context of p[ME-3921/

–3631]ME-147/+31CAT. A mutation within the COUP-TF half-site (mut G2) caused a 45% decrease in T3 regulation of promoter activity in CEH (Figure 6). This observation provides further evidence for a role of COUP-TF complexes in enhancing T3 responsiveness in CEH.

Interestingly, mutation of sequences flanking the COUP-TF half-site (mut G1 and mut G4) also inhibited T3 responsiveness. One explanation for this result is that region G contains binding sites for transcription factors other than COUP-TF and that the binding of proteins to sites distinct from the COUP-TF-binding site was not detected in the gel mobility-shift assay. Alternatively, sequences flanking the COUP-TF half-site may modulate the transcriptional activity of COUP-TF without causing changes in COUP-TF-binding affinity. In support of the latter possibility, studies analysing the structure–function relationships of T3REs have shown that variations in the structure of the upstream half-site of certain DR4 elements can profoundly alter the transcriptional activity of TR-RXR heterodimers without causing changes in TR-RXR binding affinity [32]. The mechanism mediating the alteration in TR-RXR activity involves changes in the conformation of TR-RXR heterodimers bound to DNA [33].

Characterization of proteins that bind region H

The binding of proteins to region H was analysed using gel mobility-shift assay. Incubation of a labelled oligonucleotide probe containing region H (–3663 to –3633 bp, wt H) with nuclear extracts from CEH or CEF resulted in the formation of multiple protein–DNA complexes (Figure 7B). Results from competition analyses with unlabelled oligonucleotides containing

Figure 6 Effects of site-specific mutations of regions G and H on T3 regulation of transcription

p[ME-3921/-3631]ME-147/+31CAT or constructs containing mutations of region G or region H in the context of p[ME-3921/-3631]ME-147/+31CAT were transiently transfected into CEH as described in the Materials and methods section. Transfections also contained CMV- β -galactosidase as a control for transfection efficiency. After the transfection, cells were treated with or without T3 for 48 h. Cells were then harvested, extracts prepared, and CAT and β -galactosidase assays were performed. The mutated sequences in regions G and H are shown in Figures 2, 4 and 7. CAT activity was expressed relative to β -galactosidase activity to correct for differences in transfection efficiency. CAT activity in cells transfected with p[ME-3921/-3631]ME-147/+31CAT and incubated without T3 was set at 1, and the other activities were adjusted proportionately. Fold stimulation by T3 was calculated as described for Figure 2. The results are means $\pm$ S.E.M. from five experiments. The asterisk indicates that the fold stimulation by T3 for p[ME-3921/-3631]ME-147/+31CAT was significantly higher than that of any other construct ($P < 0.05$).

sequences that were identical (lanes 2 and 12) or unrelated (lanes 10 and 20) to the probe sequence indicated that marked differences existed between CEH and CEF in the pattern and intensity of sequence-specific protein binding to region H. In CEF, most of the protein binding to region H was competed with by the region H probe sequence but not by the unrelated sequence, suggesting that protein binding to this element was primarily sequence-specific. In CEH, however, a substantial portion of the protein binding to region H was competed with by both the probe sequence and the unrelated sequence, suggesting that the majority of protein binding to this element was not sequence-specific. Nevertheless, several sequence-specific bands were detected in CEH. Some of these bands were completely competed with by the region H probe sequence, but only partially competed with by the unrelated sequence. The apparent partial competition of region H binding by the unrelated sequence may be explained by the co-migration of protein complexes that are sequence-specific with those that are not sequence-specific.

To determine the sequences in region H that were required for sequence-specific protein binding, competition analyses were performed with unlabelled oligonucleotides containing 3 bp mutations spanning the -3663 to -3633 bp region. Oligonucleo-

Figure 7 Characterization of proteins that bind to region H

Gel mobility-shift experiments were performed as described in the Materials and methods section using a 32 P-labelled DNA probe (-3663 to -3633 bp) containing region H and nuclear extracts from CEH (lanes 1-10) and CEF (lanes 11-20). Incubations with the region H probe were performed in the absence and presence of unlabelled competitor DNAs (50-fold molar excess). DNA and DNA-protein complexes were resolved on 6% non-denaturing polyacrylamide gels. (A) The sequences of the competitor DNAs. wt H is the native malic enzyme sequence between -3663 and -3633 bp. mut H1-mut H7 contain 3 bp mutations within the -3663 to -3633 bp sequence. The sequence of the unrelated competitor DNA was 5'-CTGGTACTTCAACATGCCAC-3'. (B) Competition analysis with native and mutant competitor DNAs. These data are representative of four experiments employing independent preparations of nuclear extract.

tide competitors containing mutations at -3659/-3657 bp (mut H1), -3655/-3653 bp (mut H2), -3646/-3644 bp (mut H5), -3643/-3641 bp (mut H6) and -3639/-3637 bp (mut H7; see Figure 7A) were effective in competing for the binding of sequence-specific protein complexes in CEH and CEF (Figure 7). In contrast, mutations at -3652/-3650 bp (mut H3) and -3649/-3647 bp (mut H4) were not effective in competing for the binding of sequence-specific protein complexes in CEH and CEF. These data indicate that the region between -3652 and -3647 bp (5'-CAGGTG-3') is required for the binding of sequence-specific protein complexes in CEH and CEF. This region strongly resembles an E-box motif that binds members of the basic helix-loop-helix family of transcription factors.

In previous work, several basic helix-loop-helix factors have been shown to play a role in regulation of gene transcription in liver. These factors include upstream stimulatory factor (USF)-1 [34], USF-2 [34] and sterol regulatory element-binding protein-1 (SREBP-1) [35]. Results from gel mobility-shift experiments using antibodies against USF-1, USF-2 or SREBP-1

Chick Embryo Hepatocytes			
Construct	Relative CAT Activity		Fold Stimulation by T3
	- T3	+ T3	
T3RE-2 ME-147/+31 CAT	1.0	11 ± 2	11 ± 2
T3RE-2 Region G ME-147/+31 CAT	0.6 ± 0.1	14 ± 2	23 ± 2*
T3RE-2 Region H ME-147/+31 CAT	0.7 ± 0.1	17 ± 2	25 ± 3*
T3RE-2 mut G block ME-147/+31 CAT	1.0 ± 0.2	8 ± 2	8 ± 1

Figure 8 Regions G and H can function alone to enhance T3 regulation of transcription

Oligonucleotides containing region G (−3682 to −3662 bp), region H (−3661 to −3633 bp) or a block mutation of region G in the context of the −3682 to −3662 bp fragment (mut G block) were inserted downstream of malic enzyme T3RE-2 in p[T3RE-2]ME-147/+31CAT. Hepatocytes were transiently transfected with these constructs and treated with or without T3 as described in the Materials and methods section. CAT activity in cells transfected with p[T3RE-2]ME-147/+31CAT and treated without T3 was set at 1, and the other activities were adjusted proportionately. Fold stimulation by T3 was calculated as described for Figure 2. The results are means ± S.E.M. from five experiments. Asterisks indicate that the fold stimulation by T3 was significantly higher than that of cells transfected with p[T3RE-2]ME-147/+31CAT or p[T3RE-2 + mut G block]ME-147/+31CAT ($P < 0.05$).

indicated that none of these factors bound region H in CEH and CEF (results not shown). The lack of binding of USF-1 and USF-2 to region H was supported by the observation that an oligonucleotide containing the consensus binding site for USF was not effective in competing for sequence-specific protein binding to region H in CEH and CEF (results not shown). The sequence of the E-box motif in region H (5′-CAGGTG-3′) differs from the sequence of the USF consensus binding site (5′-CACGTG-3′) at only one position. Thus the G residue at position 3 of the E-box motif in region H appears to be an important determinant of sequence-specific protein binding in CEH and CEF.

Transient-transfection experiments were performed to determine whether the E-box motif in region H was required for optimal T3 responsiveness in CEH. Mutation of the last 3 bp of the E-box motif (i.e. mut H4) in the context of p[ME-3921/−3631]ME-147/+31CAT caused a 55% decrease in T3 regulation of promoter activity (Figure 6). This observation provides further support for a role of an E-box-binding protein in augmenting T3 responsiveness in CEH. Mutation of sequences flanking the E-box in region H also decreased T3 regulation of transcription in CEH. These sequences may modulate T3 responsiveness by interacting with proteins not detected in the gel mobility-shift assay or by altering the conformation of proteins bound to the E-box motif without affecting their DNA-binding affinity.

Regions G and H can function alone to enhance T3 regulation of transcription directed by a T3RE

Proteins bound to regions G and H may enhance T3 responsiveness in CEH by interacting with TR-RXR heterodimers bound to the adjacent T3RU at −3878/−3769 bp. Alternatively, proteins bound to regions G and H may enhance T3 responsiveness by interacting with other TR accessory proteins bound to sequences flanking the T3RU. To investigate whether TR-RXR interacts directly with proteins bound to regions G and H, transient-transfection experiments were conducted to determine whether region G or region H could act alone to enhance T3

regulation of transcription directed by a T3RE. Within the malic enzyme, T3RU is a strongly active T3RE designated as T3RE-2 (−3878/−3863 bp). When CEH were transfected with a reporter plasmid containing T3RE-2 linked to the minimal promoter of the malic enzyme gene (p[T3RE-2]ME-147/+31CAT), T3 stimulated an 11-fold increase in promoter activity (Figure 8). When a DNA fragment containing region G (−3682 to −3662 bp) or region H (−3661 to −3633 bp) was inserted downstream of T3RE-2 in p[T3RE-2]ME-147/+31CAT, the stimulation of promoter activity caused by T3 was increased by approx. 2-fold. Insertion of a DNA fragment containing mut G block downstream of T3RE-2 in p[T3RE-2]ME-147/+31CAT had no effect on T3 responsiveness, indicating that the increase in T3 responsiveness caused by regions G and H was not due to changes in the spacing between T3RE-2 and the minimal malic enzyme promoter. These data suggest that proteins bound to regions G and H in CEH enhance T3 regulation of transcription, at least in part, by interacting with TR-RXR heterodimers bound to the T3RU.

DISCUSSION

In this report, we have identified two new TR accessory elements that flank the malic enzyme T3RU. One element, designated region G, contains a single nuclear-hormone-receptor half-site that binds COUP-TF. The other element, designated region H, contains an E-box motif that interacts with proteins that are distinct from USF and SREBP. The identification of regions G and H expands the total number of accessory elements flanking the malic enzyme T3RU to five. These accessory elements are arranged 5′ to 3′ as region A (−3895/−3890 bp), region E (−3761/−3744), region F (−3703/−3686 bp), region G (−3681/−3666 bp) and region H (−3655/−3646 bp; Figure 1). Each of these TR accessory elements enhances T3 regulation of transcription in CEH but not in CEF, suggesting that they play a role in mediating cell-type-dependent differences in T3 responsiveness. An interesting feature of region H that distinguishes it from the other TR accessory elements is that it functions not only as an enhancer of T3 responsiveness in CEH, but also as a T3-insensitive repressor of transcription in CEF (Figure 2). The T3-insensitive repressor activity of region H in CEF may contribute to the low rate of T3-induced malic enzyme transcription in CEF relative to that observed in CEH [11]. Differences in region H activity between CEH and CEF may be mediated by qualitative and quantitative alterations in protein binding to this element (Figure 7).

Previous studies have shown that COUP-TF is a potent repressor of T3 action and that this repressor activity plays an important role in the regulation of developmental processes in brain and muscle [31,36,37]. Four mechanisms have been proposed to explain the inhibitory effects of COUP-TF on T3 signalling. First, COUP-TFs bind to a number of T3REs and thus may compete with TR for binding to these DNA elements [38]. Secondly, COUP-TFs heterodimerize with RXR and thus may compete with TR for heterodimerization with RXR [39]. Thirdly, COUP-TFs may inhibit T3-induced transcription by binding directly to the ligand-binding domain of TR [40]. Finally, COUP-TFs have been shown to repress basal and T3-induced promoter activity by binding specific DNA elements and recruiting co-repressors such as nuclear-receptor co-repressor and silencing mediator for RAR and thyroid hormone receptor [40,41]. In addition to the wealth of data documenting the inhibitory effects of COUP-TF on T3 action, the results of the present study are the first to show that COUP-TF can function as an activator of T3 signalling. COUP-TF bound to region G

stimulates T3 responsiveness in CEH by augmenting the repressor activity of TR in the absence of T3 and the de-repression/activation activity of TR in the presence of T3 (Figures 2, 6 and 8). COUP-TF may enhance TR activity by facilitating the recruitment of TR co-repressors in the absence of T3 and TR co-activators in the presence of T3. Alternatively, COUP-TF may augment TR activity by modulating interactions between TR and the basal transcription factor, TFIIB. Previous studies have shown that both TR and COUP-TF interact physically with TFIIB [42–44]. Interactions between COUP-TF and TFIIB and/or COUP-TF and TR may alter the binding of TR to TFIIB in the absence or presence of T3.

Theoretically, promoter-specific differences in the ability of COUP-TF to activate or inhibit TR activity expand the dynamic range of T3 signalling in the cell. This phenomenon may be due to variations in the DNA sequences comprising the COUP-TF-binding sites. Previous studies have shown that variations in the sequence of a *cis*-acting element can modulate the activity of a bound transcription factor via an allosteric mechanism [45]. The COUP-TF-binding site in region G is unusual in that it consists of a single AGGTCA half-site motif rather than two half-sites arranged as direct or inverted repeats. Thus the conformation of COUP-TF bound to region G may be different from the conformation of COUP-TF bound to other elements containing a repeated half-site structure. DNA-induced changes in the conformation of COUP-TF may alter the ability of this transcription factor to recruit TR co-regulatory proteins or modulate interactions between TR and TFIIB.

Previous studies have shown that COUP-TF can function as a positive accessory factor for other hormone signalling pathways. For example, retinoic acid-induced stimulation of the RAR β promoter is augmented by the binding of COUP-TF to a DNA element containing directly repeated half-sites separated by an 8 bp spacer [46]. COUP-TF stimulates RAR β promoter activity by interacting physically with RAR α , bound at an adjacent site. This interaction facilitates the recruitment of CREB-binding protein to RAR α . Other studies have shown that optimal stimulation of the phosphoenolpyruvate carboxykinase (PEPCK) promoter by glucocorticoids is dependent on the presence of two COUP-TF-binding sites that flank two glucocorticoid receptor-binding sites [47,48]. These COUP-TF-binding sites, termed AF-1 and AF-3, require the presence of at least one other accessory element in the PEPCK promoter in order to be effective in stimulating glucocorticoid responsiveness. Interestingly, the AF-1 site is also located near a T3RE in the PEPCK promoter, but is not effective in modulating T3 regulation of PEPCK transcription in hepatoma cells [49,50]. This observation illustrates further the role of promoter context in determining the effects of COUP-TF on hormone signalling.

Data from gel mobility-shift analyses indicate that region G binds multiple COUP-TF complexes and that the abundance of these complexes varies between CEH and CEF (Figures 3–5). These alterations in protein binding to region G may play a role in mediating differences in region G activity between CEH and CEF. We propose that complex 3, the predominant complex observed in CEH, is effective in enhancing TR activity, whereas complexes 1 and 2, the predominant complexes observed in CEF, are not effective in modulating TR activity. Complexes 1–3 may contain different forms of COUP-TF and/or proteins that dimerize with COUP-TF. Differential dimerization of COUP-TF with RXR or TR is not responsible for the different region-G complexes, since RXR and TR were not detected in any of these complexes using antibody supershift analysis (Figure 2).

A feature of the malic enzyme gene that distinguishes it from other T3-responsive genes is the large numbers of T3REs (i.e. six)

and accessory elements (i.e. five) that are required for optimal T3 regulation of transcription in CEH. We postulate that TR-RXR heterodimers and TR accessory proteins bound to the malic enzyme enhancer at –3910/–3640 bp assemble into a higher-order transcription factor–enhancer DNA complex, termed an enhanceosome [51]. Interactions between the proteins comprising this enhanceosome may facilitate the recruitment of co-repressors in the absence of T3 and co-activators in the presence of T3. Such a scenario would explain the high level of T3 responsiveness of the malic enzyme gene relative to other T3-regulated genes that lack complex T3 regulatory structures. The ability of the enhanceosome at –3910/–3640 bp to confer cell-type-dependent differences in T3 regulation of malic enzyme transcription is probably mediated by qualitative and/or quantitative alterations in protein binding to the T3RU [15,19], region A [19], region F [19], region G (Figure 3) and region H (Figure 7). Data from transfection analyses indicate that sequences located within the T3 enhancer region at –3910/–3640 bp are involved in conferring the stimulation of malic enzyme transcription by glucose and the inhibition of malic enzyme transcription by cAMP and medium-chain fatty acids ([52], and Y. Wang and F. B. Hillgartner, unpublished work). A role for this region of the malic enzyme gene in mediating the actions of glucose, cAMP and medium-chain fatty acids is supported further by the observation that these compounds require the presence of T3 to be effective in regulating malic enzyme transcription [6,7,10]. Thus the enhanceosome at –3910/–3640 bp is an integration point for multiple signalling pathways controlling malic enzyme transcription. Future studies will investigate the mechanism by which the enhanceosome at –3910/–3640 bp mediates the effects of hormones and nutrients on malic enzyme transcription in hepatocytes.

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