The hormonal regulation of carbamoylphosphate synthetase I expression
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The role of proximal enhancer elements in the regulation of carbamoylphosphate synthetase expression

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**ABSTRACT**

As part of the urea cycle, carbamoylphosphate synthetase (CPS) converts toxic ammonia resulting from amino-acid catabolism into urea. Liver-specific and glucocorticoid-dependent expression of the gene involves a distal enhancer, a promoter-proximal enhancer, and the minimal promoter itself. When challenged with glucocorticoids, the glucocorticoid-responsive unit (GRU) in the distal enhancer of the carbamoylphosphate-synthetase gene can only activate gene expression if, in addition to the minimal promoter, the proximal enhancer is present. Here, we identify and characterise two elements in the proximal CPS enhancer that are involved in glucocorticoid-dependent gene activation mediated by the GRU. A purine-rich stretch forming a so-called GAGA-box and a glucocorticoid-response element (GRE) are both crucial for the efficacy of the GRU and appear to constitute a promoter-proximal response unit that activates the promoter.
**INTRODUCTION**

In the liver, the mitochondrial enzyme carbamoylphosphate synthetase I (CPS; E.C. 6.3.4.16) is a key enzyme in the ornithine cycle, which functions to convert toxic ammonia resulting from amino-acid degradation into urea. Apart from liver, CPS is only expressed in the enterocytes of the small intestine, where it plays a key role in the synthesis of citrulline.

The regulatory regions for hepatic CPS expression reside within a minimal promoter region containing the TATA-box, a 123-basepairs proximal enhancer (-161 to -38), and in a 469-basepairs distal enhancer located 6.3 kb upstream of the transcription-start site (Figure 1, panel A). The proximal enhancer contains three C/EBP-like sequences and a 28-basepairs purine-rich stretch forming a so-called GAGA-box. The 469-basepairs distal enhancer can activate tissue-specific and hormone-sensitive gene transcription in conjunction with the proximal enhancer and minimal promoter. An 80-basepairs subregion of the 469-basepairs distal enhancer relays about 50% of this tissue-specific and hormone-sensitive expression. This region contains binding sites for the ubiquitously expressed glucocorticoid receptor (GR), the liver-enriched transcription factors CCAAT/enhancer binding protein (C/EBP), forkhead box A (FoxA/HNF3), and an unknown protein denoted P3. Since mutation of either of these transcription factor-binding sites abolishes the glucocorticoid response, this 80-basepairs region forms a glucocorticoid-responsive unit (GRU).

Enhancer elements often function to provide spatio-temporal activation of gene promoters, the activity of which is otherwise weak. In most instances, however, the specificity of this interaction depends on the ability of the enhancer to physically communicate with the autologous promoter. For instance, in the α-fetoprotein promoter a promoter-coupling element is essential for linking its distal enhancers to the promoter. Many enhancers have been shown to function with heterologous promoters, but to a much lesser extent than with their autologous promoter. This is apparent in the regulation of CPS expression, where the 469-basepairs distal CPS enhancer shows a reduced hormonal inducibility when placed in front of the thymidine kinase (TK) or minimal CPS promoter, and cannot activate transcription from the Drosophila heatshock protein-70 gene promoter. This implies that additional regulatory sequences are required that are lacking in these heterologous promoters.

In this study, we show that the CPS proximal enhancer fulfills the role of relaying the activating signal of the GRU onto the transcription machinery. We
furthermore evaluate the role of the individual response elements in the proximal enhancer of the CPS gene. The GAGA-box and a newly identified proximal GRE are indispensable, whereas the C/EBP-like sequences in the proximal enhancer do not contribute to reporter gene activation in response to glucocorticoids.

**METHODS**

**Tissue culture**

FTO-2B rat hepatoma cells and COS-1 cells were grown in DMEM/F12 medium (Invitrogen life technologies, Carlsbad, CA, USA), supplemented with 10% foetal calf serum, in a 5% CO₂ atmosphere at 37°C. FTO-2B cells have constitutive protein kinase A activity because of an absence of the regulatory Rλ subunit.

**Oligonucleotides and plasmids**

All constructs described in this paper were cloned into the vector pSPluc+ (Promega, Madison, WI, USA), containing the bovine growth-hormone polyA tail in between the Xbal-EcoRV sites downstream of the luciferase gene. Subsequently, the minimal promoter with or without the proximal enhancer was inserted into the Kpnl-HindIII sites of the polylinker. GRU constructs were cloned into the BamHI and PstI sites directly upstream of the minimal promoter or proximal enhancer when present.

**Transfections**

1·10⁷ FTO-2B hepatoma cells were electrotransfected with 20 μg of tester plasmid DNA and 2 μg pRL-CMV (Promega, Madison, WI, USA) to correct for differences in transfection efficiency. Transfected cells were split into two equal parts and cultured in two 9.6 cm² wells. 24 hours posttransfection the medium was replaced and cultured for another 24 hours; in one of the two wells this medium was supplemented with 100 nM dexamethasone (Centrafarm, Etten-Leur, The Netherlands). Luciferase activity was measured using the dual-luciferase reporter assay system (Promega, Madison, USA) in an Autolumat plus (Berthold, Vilvoorde, Belgium). Luciferase values were corrected for differences in transfection efficiency and between-session variation. Transfection results are presented as means of at least four experiments and their SEM. Fold induction is given as the ratio between glucocorticoid-induced and basal reporter gene expression. To analyse the differences between constructs, the non-parametric Kruskal-Wallis test was employed. Results that are significantly different (p<0.05) from the wild-type GRU in
conjunction with the proximal enhancer and minimal promoter are marked by an asterisk.

**Preparation of nuclear extracts from COS-1 cells**
COS-1 cells were transfected with expression vectors for C/EBPα using polyethyleneimine as transfection agent. Two days posttransfection, cells were harvested and resuspended in lysis buffer (10 mM Tris pH 7.4; 15 mM NaCl; 60 mM KCl; 1 mM EDTA; 0.1 mM EGTA; 0.1% Triton X100; 150 mM sucrose; 0.15 mM spermine; 0.5 mM spermidine) precoolled to 4°C. Nuclei were sedimented through a 1 M sucrose cushion for 20 minutes at 1400g and resuspended in one volume of low salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT). After resuspension, one volume of high salt buffer (low-salt buffer, in which the 20 mM KCl was substituted by 800 mM KCl) was added drop-wise, and the nuclei were extracted by incubation for 20 minutes at 4 °C on a tilt board. The extract was dialysed against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, frozen in liquid nitrogen and stored at −80 °C.

**Antibodies and GR DNA-binding Domain**
Rabbit polyclonal antibodies against C/EBPα (cat# sc-61) were purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA). The GR DNA-binding domain was kindly provided by Dr. K.R. Yamamoto, San Francisco, CA, USA.

**Electrophoretic mobility-shift assay**
Complexes of protein and labeled DNA were resolved on a 6% polyacrylamide gel (acrylamide:bisacrylamide=29:1) in 0.25x TBE buffer (1x TBE: 45 mM Tris, 45 mM boric acid, 1 mM EDTA) at room temperature. Before loading the samples, gels were pre-run for 1 hour. The probe was radiolabeled using a PCR with [α-³²P]dATP and purified over a Qiaquick column (Qiagen, Valencia, CA, USA). After digestion to remove sequences flanking the response element (RE), the probe was purified using a Sephadex G50 column. Each binding reaction contained 10 µg nuclear extract, 20 mM HEPES pH 7.9, 1 µg poly(dl-dC)-poly(dl-dC), 10% glycerol, 100 mM KCl in a final volume of 20 µl. After 10 minutes preincubation, the probe was added (2·10⁴ cpm) and complexes were allowed to form for 20 minutes. For C/EBP, this incubation step was conducted on ice, whereas for GR, this was performed at room temperature. To perform competition experiments, cold (non-)specific
oligonucleotides were added to the reaction mixture, whereas for supershift analyses, 1 µl antiserum was added 15 minutes after commencing the binding reaction. Samples were loaded on gel without the use of dye. Following electrophoresis at 10 V·cm⁻¹ until the bromophenol blue reference migrated two-third the length of the gel, it was dried and exposed overnight to phosphorus screen and analysed using a Storm 860 PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA, USA).

RESULTS

The proximal enhancer is essential for the GRU-mediated glucocorticoid-response

To establish which regulatory regions of the CPS gene are required for glucocorticoid-mediated gene expression, we tested combinations of these regions in luciferase-reporter constructs.

Figure 1. Both the proximal and distal enhancers of the CPS gene are required for glucocorticoid-dependent transcription. A scheme of the regulatory regions of the CPS gene is shown in panel A. Panel B shows the data from transient transfections into FTO-2B hepatoma cells after 24 hours induction with glucocorticoids (±SEM). The asterisk indicates significant differences relative to construct d. The data show that the minimal promoter alone is inactive (a). Neither the proximal enhancer (b) nor the distal GRU (c) alone can mediate a glucocorticoid response from the minimal promoter. Only the combination of the GRU, the proximal enhancer and the minimal promoter (d) does mediate a strong glucocorticoid response.
Transient transfection of the minimal promoter or the minimal promoter in conjunction with the proximal enhancer did not generate any reporter-gene expression (Figure 1, constructs a, b). Construct c shows that the GRU was not able of activating reporter gene expression from the minimal promoter. Only the combination of the three regulatory units was able to drive reporter-gene expression when induced with glucocorticoids. (Figure 1, construct d). These data thus show that the proximal enhancer contains sequences that are essential for relaying the activating signal of the GRU onto the promoter.

**C/EBP-like sequences in the proximal enhancer do not contribute to glucocorticoid-mediated reporter-gene expression**

To determine which elements in the rat CPS proximal enhancer are required for the activity of the GRU, we optimised and mutated putative response elements in the proximal enhancer.

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**Figure 2.** The C/EBP-like sequences in the proximal enhancer have no influence on a GRU-mediated glucocorticoid response. C/EBP-like sequences (hatched ovals) in the proximal enhancer were either replaced by optimal C/EBP sequences (spotted ovals) or mutated (indicated by a cross). These modified proximal enhancers were cloned between the distal GRU and minimal promoter of the CPS gene in luciferase-reporter constructs. The DNA constructs were transiently transfected to FTO-2B hepatoma cells. After 24 hours induction with glucocorticoids, luciferase values were measured. Neither optimisation (b) nor inactivation by mutation of the C/EBP-binding sequences in the proximal enhancer (c, d, e) resulted in a significantly different activity compared to the parent construct (a).
Optimisation of the C/EBP-like sequences in the proximal enhancer by replacing them all with a C/EBP sequence selected for optimal binding \(^1\) increased the activity in response to glucocorticoids only by 1.4-fold. (Figure 2, constructs a and b). Basal activity was also increased, causing the fold-induction to be lower in this construct. Since the moderate effect of optimisation of the C/EBP-like response elements could be due to their already near-optimal sequence, we mutated these sites. Whereas such mutations lowered basal activity, they did not lead to a significant decrease in glucocorticoid-induced activity, irrespective of whether one, two, or all three C/EBP sites were mutated (Figure 2, constructs c, d, e). These C/EBP-like sequences, therefore, have a limited effect on basal activity, but do not play any role in the GRU-mediated gene activation by glucocorticoids.

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**Figure 3. Mutation of the C/EBP sequence in the proximal enhancer abolishes C/EBP binding.** Mobility-shift assays with wild type and mutated C/EBP-like binding sites were conducted. The S3 C/EBP-binding site of the TAT gene (tgtttacgctcctctcactctct) was labeled and incubated with a C/EBP\(\alpha\)-enriched COS-cell nuclear extract. The complex was separated on a native polyacrylamide gel. Incubation together with 0.5 \(\mu\)l C/EBP\(\alpha\) antiserum gave rise to a supershift (Panel A). Panel B shows a competition experiment in which 0, 0.1, or 1 pmol ds competitor DNA was added to the reaction mixture. The numbers at the bottom of the gel indicate the intensity of the specific complex relative to the sample without competitor. The wild type C/EBP-like sequence in the proximal enhancer (gtccatttacgctcctctctct) competes with the S3 C/EBP oligonucleotide for C/EBP-binding (Panel B, lanes a, b, c), whereas the mutated C/EBP-binding site (gtccatttacgctcctctctct) did not (Panel B, lanes d, e, f).
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To assure that the mutations in the C/EBP-like sequences indeed prevented binding of C/EBP, we analysed protein-DNA interactions of these elements with C/EBPα-enriched nuclear extract in mobility-shift assays. The radiolabeled S3 C/EBP RE from the tyrosine aminotransferase (TAT) gene specifically bound C/EBPα, as shown by the supershift in the presence of C/EBP antibodies (Figure 3, panel A). The EMSA in Figure 3 shows that the wild-type C/EBP-like sequence from the proximal enhancer competed efficiently with the S3 C/EBP probe for protein binding, whereas the mutated C/EBP probe did not (Figure 3, panel B). These data demonstrate that our mutations of the C/EBP-like sequences severely impair C/EBP binding and underscore our earlier conclusion that C/EBP plays no role in GRU-mediated gene activation.

The proximal enhancer contains a GRE that is indispensable for the GRU-mediated glucocorticoid response

A transcription factor-database search identified two putative GREs in the proximal enhancer, sandwiched between the three C/EBP-like sequences (Figure 4, panel A).

A.

Consensus GRE: GGTACAn nTGTTCn

catgatttcaacagggagaactgcattttcatgtgcatttgatactgtgacatca ggtgcattt ggttcattgctat

gtactaaacttctccttgaccttgacatcacatttgactagttg ACA

Figure 4. The putative GREs in the proximal enhancer are required for the GRU-mediated glucocorticoid response. Panel A indicates the location of the two putative GREs in the proximal enhancer. To test the functionality of these REs, mutations were introduced into these REs. The endogenous GRE-1 sequence (TGTATGacaTGTCCA) was replaced by TACTTGacaTGTCCA, while the GRE-2 sequence (GGTGCCattTGCTAT) was replaced by GGAGTTattAGGAAG. This modified proximal enhancer was tested in combination with the distal GRU and minimal promoter by transient transfection to FTO-2b hepatoma cells. Panel B shows that mutation of the proximal-enhancer GREs (b) significantly (asterisk) reduces glucocorticoid-dependent reporter-gene activity relative to its parent construct (a).
Luciferase reporter constructs were made containing the GRU, a modified proximal enhancer in which the putative GREs were mutated, and the minimal promoter. Transient transfection with this double mutant showed only 10% reporter-gene activity in response to glucocorticoids (Figure 4, panel B), indicating that it is a key element in relaying the activating signal of the induced GRU onto the transcription machinery. To establish whether GR indeed binds to both REs, we conducted a mobility-shift assay using the DNA-binding domain of the glucocorticoid receptor (GR-DBD) and a radiolabeled GRE probe as suggested by Santa Cruz biotechnology (Santa Cruz, USA). Binding of the probe to GR-DBD resulted in a specific complex, since it could be competed for by a cold GRE (Figure 5, lanes b, c, d, e). In this assay, GRE-2 of the proximal promoter did not function as an efficient competitor (lanes f, g, h), whereas the GRE-1 (lanes i, j, k) was as effective as the Santa-Cruz GRE (lanes c, d, e). These data show that only GRE-1 in the CPS proximal enhancer is a bona-fide GRE.

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**Figure 5. The DNA-binding domain of GR binds to GRE-1 in the proximal enhancer.** A radiolabeled consensus GRE probe was incubated with the GR DNA-binding domain and separated on gel. The EMSA shows the free probe and the DNA-GR complex (lane b). Addition of increasing amounts (0.1, 1, and 10 pmol) of consensus GRE (gagatcTGTACAggaTGTCTTagatga) competes with the radiolabeled GRE for binding to GR DNA-binding domain (lanes c,d,e). Whereas GRE-2 (gcagaa GGTGCCattTGCTAgtaggat) cannot compete with the probe (lanes f,g,h), GRE-1 (gcagctTGTCTGacaTGTCCA ttggagg) can (lanes i,j,k). The numbers at the bottom indicate the intensity of the specific complex relative to sample without competitor (lane b).
The GAGA-element in the proximal enhancer is required for a GRU-mediated glucocorticoid response

A third feature of the proximal enhancer is the GAGA-box located only a few nucleotides upstream of the TATA-box. A construct containing the GRU, the proximal enhancer with mutated GAGA-box, and the minimal promoter exhibited only 10% of the glucocorticoid-induced activity relative to the parent construct (Figure 6, constructs a, b). Also, fold-induction by glucocorticoids was reduced from 54-fold in the parent construct to 14-fold in the construct with an inactive GAGA-box. These data show that, within the proximal enhancer region, the GAGA-box is the second essential element to qualitatively regulate GRU-dependent promoter transactivation.

Figure 6. The GAGA-box is an essential proximal-enhancer element for GRU-mediated gene transcription. The wild type GAGA sequence (cttgagggaggctggagggaggag) in the proximal enhancer was replaced by a mutated oligonucleotide (cttggcagcagttgctgtggagccgatgag) and tested in conjunction with the distal GRU and minimal promoter. Transient transfections to FTO-2B hepatoma cells followed by a 24 hours induction by glucocorticoids, show that mutation of the GAGA-box (b) results in a very low activity that is significantly different from its parent construct (a).

DISCUSSION

Many enhancers have been shown to act in conjunction with a heterologous promoter, but very often the activity and/or the regulation of expression in these constructs is affected. This indicates that the sequences surrounding the minimal promoter contain information that mediates the activating signal of the enhancer. From the experiments described in this paper, it has become apparent that the proximal enhancer of the CPS gene is an essential element for relaying the activating signal of the GRU to the promoter. The strict requirement for the proximal enhancer suggested that it acts as a switch and might, therefore, contain response element(s) that are required for glucocorticoid responsiveness. This study shows that a GRE and a GAGA-box function as the regulatory elements in the proximal enhancer that mediate this effect.
The C/EBP-like sequences in the proximal enhancer do not contribute to glucocorticoid-dependent gene expression

In the PEPCK gene, a distally located cluster of response elements acts in conjunction with a proximal C/EBP-binding site to form a functional GRU \(^19\). Since the GRU of the CPS gene is also located far upstream and requires the presence of the proximal enhancer to be functional, our attention was drawn to the C/EBP-like sequences within this proximal enhancer. Although optimisation or mutation of these three C/EBP-like sequences in the proximal enhancer mildly affected basal activity, it had no noticeable effect on the glucocorticoid-induced activity. The finding that C/EBP\(\alpha\) could bind to C/EBP-like sequences, but not to the mutated sequences further demonstrated that these sequences do not function as C/EBP REs.

Previously, a weakly positive effect of the C/EBP II element in the proximal enhancer on reporter gene expression was reported \(^5,20\). However, these studies addressed promoter behaviour in the absence of the GRU. In a later study, Goping and colleagues did study the function of the proximal enhancer elements in conjunction with the distal enhancer, but in absence of glucocorticoids, that is, when the GRU is only minimally active \(^21\).

The proximal GRE in the proximal enhancer is crucial for GRU-mediated gene expression

Transcription factor-database searches with the sequence of the proximal enhancer region revealed the presence of two putative GREs sandwiched between the C/EBP-like sequences. A consensus GRE has been defined as GGTACAnnnTGTTCT with the downstream half (TGTTCT) being the most important, since initial contact by the GR monomer occurs at that site \(^22\). Both putative sites have a moderate sequence homology with the consensus GRE sequence, as is reflected by the matrix-similarity scores (ranging from 0.5 to 1.0) of 0.88 and 0.73 for GRE-1 and GRE-2, respectively. However, GREs can be far from perfect and still be functional as is also the case for the highly responsive GRE in the CPS GRU. In agreement, the glucocorticoid-response became severely impaired when these sequences were mutated. Since only the proximal GRE-1 can bind the GR DNA-binding domain, this site represents the active GRE.

Since the minimal promoter does not respond to glucocorticoids, and the combination of the proximal enhancer and the minimal promoter exerts less than 5% of the glucocorticoid inducibility exerted by the combination of the proximal and distal enhancer (Figure 1, constructs a, b), the GRE in the proximal enhancer has only a
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weak capacity to transactivate gene expression. Rather than acting as a classical transactivator of gene expression, the promoter-proximal GRE may, therefore, function to facilitate the interaction of other transcription factors with the transcription machinery.

The GAGA-box in the proximal enhancer increases the rate of gene expression

Our data demonstrate a crucial role for the GAGA-box in eliciting a glucocorticoid response, since mutation of this element reduces the glucocorticoid-dependent induction of reporter-gene activity to a similar extent as mutation of the promoter-proximal GRE. GAGA-boxes have also been described in genes for the serine protease inhibitor-2.1 (Spi-2)\textsuperscript{4}, the vasopressin receptor of the V1b subtype (V1bR)\textsuperscript{23}, and the insulin-like growth factor-I (IGF-I)\textsuperscript{24}. The CPS and Spi-2 proximal enhancers are surprisingly similar in composition. Besides the presence of a functionally equivalent GAGA-box (CPS: -67 to -40 bp; Spi-2: -64 to -37 bp)\textsuperscript{4}, they also contain sequences resembling C/EBP-response elements and a GRE. Although the CPS proximal enhancer contains three regions that can bind C/EBP, we could not establish a functional role for these sites. Similarly, the Spi-2 gene contains 5 putative C/EBP-binding sites\textsuperscript{25}, none of which could be shown to play a role in the transcription of the gene in vivo\textsuperscript{26}.

The Stat5B-binding sites that mediate the response of Spi-2 to growth hormone are positioned in close proximity (-124 to -132 bp and -139 to -147 bp) to the Spi-2 minimal promoter. This finding argues against the hypothesis that coupling the distal enhancer to the promoter is a primary function of the GAGA-box in the CPS gene\textsuperscript{24,26}.

The GRE in the CPS gene (-107 to -93 bp) and in the Spi-2 gene (-88 to -74 bp) are both functionally indispensable. The Spi-2 GRE is differentially reactive to DNaseI as a function of the level of gene transcription. Since glucocorticoids do not activate Spi-2 transcription per se, but strongly potentiate growth-hormone action\textsuperscript{27}, the GRE and the GAGA-box may well function as a (glucocorticoid-dependent) response unit with cooperative binding properties.

The rate of transcription of the Spi-2 gene in vivo correlates with the occupancy of the GAGA-box by the GAGA-binding proteins p38 and p40. Although binding of GAGA-binding proteins and the subsequent activation of transcription can occur independent of growth hormone, mutation of the GAGA-box in the Spi-2 gene greatly reduces both basal and hormone-induced promoter activities\textsuperscript{25}. The GAGA-box binding-proteins p38 and p40 are heterogeneous nuclear ribonucleoproteins
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(hnRNPs) that maybe involved in the recruitment of a promoter-specific set of general transcription factors in the basal transcription machinery\textsuperscript{4}, because related hnRNPs, such as hnRNP-D0 and CBF-A, have been shown to be regulators of gene expression\textsuperscript{28,29}.

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REFERENCES


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