Structural requirements of the glucocorticoid-response unit of the carbamoyl-phosphate synthase gene

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The GRU (glucocorticoid-response unit) within the distal enhancer of the gene encoding carbamoyl-phosphate synthase, which comprises REs (response elements) for the GR (glucocorticoid receptor) and the liver-enriched transcription factors FoxA (forkhead box A) and C/EBP (CCAAT/enhancer-binding protein), and a binding site for an unknown protein denoted P3, is one of the simplest GRUs described. In this study, we have established that the activity of this GRU depends strongly on the positioning and spacing of its REs. Mutation of the P3 site within the 25 bp FoxA–GR spacer eliminated GRU activity, but the requirement for P3 could be overcome by decreasing the length of this spacer to ≤ 12 bp, by optimizing the sequence of the REs in the GRU, and by replacing the P3 sequence with a C/EBPβ sequence. With spacers of ≤ 12 bp, the activity of the GRU depended on the helical orientation of the FoxA and GR REs, with highest activities observed at 2 and 12 bp respectively. Elimination of the 6 bp C/EBP–FoxA spacer also increased GRU activity 2-fold. Together, these results indicate that the spatial positioning of the transcription factors that bind to the GRU determines its activity and that the P3 complex, which binds to the DNA via a 75 kDa protein, functions to facilitate interaction between the FoxA and glucocorticoid response elements when the distance between these transcription factors means that they have difficulties contacting each other.

Key words: architecture, carbamoyl-phosphate synthase, enhancer, glucocorticoid-response element, glucocorticoid-response unit.

INTRODUCTION

In liver, the enzymes of the urea cycle are responsible for the disposal of toxic ammonia originating from amino acid degradation into urea. The mitochondrial enzyme CPS (carbamoyl-phosphate synthase I; EC 6.3.4.16) is the rate-determining enzyme in this cycle [1]. Together with many genes encoding gluconeogenic and amino-acid-catabolizing enzymes, the genes encoding urea-cycle enzymes are expressed in the perportal region of the liver, and are activated by glucagon (via CAMP) and glucocorticoid hormones.

The hormonal regulation of the CPS gene is imposed by a 469 bp distal enhancer located 6.3 kb upstream of the transcription start site [2]. Within this distal enhancer, an 80 bp GRU (glucocorticoid-response unit) confers hormone responsiveness and tissue specificity upon the gene (Figure 1A) [3]. Hormone-response units are clusters of transcription factor-binding sites, comprising a hormone-responsive element and a number of cis-elements (accessory factors). Such a hormone-response unit allows the regulation of transcription of the gene in space and time by integrating multiple signal pathways [4]. We previously analysed the effect of changing the sequence of the REs, and by replacing the P3 sequence with a C/EBPβ sequence. With spacers of ≤ 12 bp, the activity of the GRU depended on the helical orientation of the FoxA and GR REs, with highest activities observed at 2 and 12 bp respectively. Elimination of the 6 bp C/EBP–FoxA spacer also increased GRU activity 2-fold. Together, these results indicate that the spatial positioning of the transcription factors that bind to the GRU determines its activity and that the P3 complex, which binds to the DNA via a 75 kDa protein, functions to facilitate interaction between the FoxA and glucocorticoid response elements when the distance between these transcription factors means that they have difficulties contacting each other.

Key words: architecture, carbamoyl-phosphate synthase, enhancer, glucocorticoid-response element, glucocorticoid-response unit.

Abbreviations used: C/EBP, CCAAT/enhancer binding protein; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; CPS, carbamoyl-phosphate synthase I; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FoxA, forkhead box A; GR, glucocorticoid receptor; GRE, glucocorticoid-response element; GRU, glucocorticoid-response unit; HNF, hepatocyte nuclear factor; PEPCK, phosphoenolpyruvate carboxykinase; RE, response element.

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Figure 1  Introduction of restriction sites in the GRU does not affect GRU activity

To facilitate cloning, restriction sites were introduced into the GRU. (A) Nucleotide sequence of the CPS GRU and the REs therein (construct a), and the modified GRU upon the introduction of restriction sites (construct b). The triangles indicate the positions of the point mutations. The GRU constructs were cloned upstream of the minimal promoter and proximal enhancer of the CPS gene. These reporter-gene constructs were transiently transfected into FTO-2B hepatoma cells and induced with glucocorticoids for 24 h. The reporter-gene activities, measured in the resulting lysates, are presented as means ± S.E.M. luciferase values for at least four experiments and the fold induction. The asterisk indicates significantly different results relative to the wild-type construct a. The data show that the combination of proximal enhancer and minimal promoter does not result in a marked increase in activity when induced by glucocorticoids relative to basal activity (B, construct c). In contrast, both the parent and the modified GRUs exhibited a similarly high level of activity when cultured in presence of glucocorticoids (B, constructs a and b). Dex, dexamethasone.

sequence, suggesting that P3 functions to facilitate the binding of transcription factors to the GRU. These findings show that the spatial requirements of the relatively simple CPS GRU are strict.

MATERIALS AND METHODS

Tissue culture

The rat hepatoma cell line FTO-2B [8] and the monkey-kidney cell line COS-1 [9] were maintained in Dulbecco's modified Eagle's medium/F12 medium (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) supplemented with 10% (v/v) foetal calf serum at 37 °C and 5% CO₂.

Transfection of reporter constructs

To determine the influence of the architectural composition of the CPS GRU on its activity, modifications were introduced into the GRU. All GRU constructs described were cloned into the BamHI and PstI sites of the pSPluc+ vector (Promega, Madison, WI, U.S.A.). For this purpose, the pSPluc+ reporter vector was modified by inserting the bovine growth hormone poly(A) tail into the XbaI–EcoRI sites downstream of the luciferase gene, and the minimal promoter containing the proximal enhancer (−161 to −38) of the CPS gene into the KpnI–HindIII sites of the polylinker upstream of the luciferase gene. To test these constructs, 20 μg of the luciferase–reporter construct was co-transfected with 2 μg of prL-CMV control DNA (Promega) into 1 × 10⁷ FTO-2B hepatoma cells by electroporation [10]. Transfected cells were divided into two equal portions and cultured in two 9.6 cm² wells. At 24 h post-transfection, the medium was replaced and the cells were cultured for another 24 h; 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) in an Autolumat plus (Berthold, Vilvoorde, Belgium). Luciferase values were corrected for differences in transfection efficiency and between-session variations. To analyse the differences between constructs, the non-parametric Kruskal–Wallis test was employed. Results were considered significantly different at P < 0.05.

Preparation of nuclear extracts from COS-1 cells

Using poly(ethylene imine) as transfection agent [11], COS-1 cells were transfected with a C/EBPα expression vector and cultured for 2 days. 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% (v/v) foetal calf serum at 37 °C and 5% CO₂.

Nuclear extract preparation from rat liver

Nuclear extracts were prepared from rat livers according to the method of Sierra [12]. Briefly, nuclei were sedimented through a 1 M sucrose cushion for 20 min at 1400 g and resuspended in 1 vol. of low-salt buffer [20 mM Hapes, pH 7.9, 25% (v/v) glycerol, 1.5 mM MgCl₂, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT (dithiothreitol)]. Nuclear proteins were extracted for 20 min at 4 °C on a tilt board by dropwise addition of 1 vol. of high-salt buffer (same as low-salt buffer, except that the 20 mM KCl was substituted by 800 mM KCl). Following 2 × 2 h dialysis against buffer comprising 20 mM Hapes, pH 7.9, 20% (v/v) glycerol, 100 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF and 0.5 mM DTT, proteins were frozen in liquid nitrogen and stored at −80 °C.
was removed by centrifugation. Nuclear proteins were salted out by a further increase in the (NH₄)₂SO₄ concentration.

To obtain a P3-enriched protein fraction, 20 mg of nuclear extract was loaded on a MonoS HR 5/5 (Amersham Biosciences, Little Chalfont, Bucks., U.K.) cationic exchanger column using an LCC 501-plus FPLC apparatus (Amersham Biosciences). Protein fractions of 1 ml were collected by eluting the column with buffer [10% (v/v) glycerol, 20 mM Hepes, pH 7.9, 1 mM EDTA, 1 mM DTT and 0.01% Nonidet P40] containing a gradient from 100 to 600 mM KCl. The resulting fractions were tested for the presence of P3 by EMSA (electrophoretic mobility-shift assay) analysis.

**EMSA analysis**

To study protein–DNA interactions, a double-stranded DNA probe was radiolabelled with [α-32P]dATP using Klenow polymerase and purified on a Sephadex G50 spin column (Amersham Biotechnologies). Each binding reaction contained 10 µg of nuclear extract, 20 mM Hepes, pH 7.9, 1 µg of poly(dI-dC) - poly(dI-dC), 10% (v/v) glycerol and 100 mM KCl in a final volume of 20 µl. Following a 10 min preincubation on ice, the probe was added (2 × 10⁶ c.p.m.) and complexes were allowed to form for 20 min on ice. To perform competition experiments, unlabelled (non)specific oligonucleotides were added to the reaction mixture, whereas for supershift analysis 1 µl of antiserum was added 15 min after commencing the binding reaction. The resulting protein–DNA complexes were resolved on a 6% (w/v) polyacrylamide gel (acrylamide/bisacrylamide, 29:1) in 0.25 × TBE buffer (1 × TBE: 45 mM Tris, 45 mM boric acid, 1 mM EDTA) at room temperature. Before loading the samples, gels were pre-run for 1 h. Samples were loaded on to the gel without the use of dye; a Bromophenol Blue reference was loaded in a separate

RESULTS

Introduction of three restriction sites in the GRU does not influence activity

When reporter-gene constructs comprising different combinations of the minimal promoter, the proximal enhancer and the distal GRU enhancer were tested, we observed no glucocorticoid response when only the proximal enhancer and the promoter were present (Figure 1B, construct c). However, addition of the GRU rendered the construct highly glucocorticoid-responsive (construct a). To facilitate modification of this GRU, three restriction enzyme recognition sites were introduced by modifying only one nucleotide per restriction site (Figure 1A). Transfection experiments using FTO-2B hepatoma cells showed that these mutations did not significantly affect activity in response to glucocorticoids (Figure 1B, construct b).

A specific arrangement of GRU elements is required for a glucocorticoid response

When we inverted the orientation of the GRU, we observed a 2.5-fold decrease in transcriptional activity (Figure 2, construct b). Because C/EBP and GR bind their REs as dimers [14,15], it is likely that their activity is independent of their orientation. In contrast, FoxA binds as a monomer. We therefore tested the possibility of a FoxA orientation-dependent glucocorticoid response. Inversion of the orientation of the FoxA site within the GRU indeed led to a 4.5-fold decrease in reporter-gene activity (Figure 2, construct c). We also tested whether the GRU REs are restricted to specific positions within the GRU by displacing the REs. Displacement of the C/EBP RE downstream of the GRE resulted in a markedly decreased activity in response to glucocorticoids (Figure 2, construct d). Together, these data indicate that the arrangement of the REs is crucial for GRU activity.

Elimination of the C/EBP–FoxA spacer increases GRU activity

The rotational phasing of a transcription factor on the DNA helix can affect its function [16,17]. Since a functional GRU is highly dependent on the presence of C/EBP [3] and its position relative to the FoxA and GR REs (Figure 2), we tested whether the distance between the GRU elements or their helical orientation affected the glucocorticoid response. When two or three helical turns (20 and 30 bp respectively) were added to the spacer between the C/EBP and FoxA REs (Figure 3, constructs e and f), activity was comparable with that of the parent construct (Figure 3, construct c). When half a helical turn was added to this region, GRU activity also remained unaffected (construct d). However, when the spacer between the C/EBP and FoxA elements was gradually eliminated, GRU activity increased 2-fold (constructs a and b).

The FoxA–GRE distance is critical for GRU activity

As shown in Figure 1(A), the FoxA and GR REs are separated by a 25 bp region that harbours a binding site for an unknown protein, denoted P3 [3]. When this element was mutated, GRU activity was all but eliminated (Figure 4, construct i). The effects of this mutation could be overcome by decreasing the spacing between the FoxA and GR REs to 12 bp (Figure 4, constructs e–h). Interestingly, the length of the FoxA–GR spacer, rather than the sequence, was important once the FoxA–GR spacer was 12 bp
The specific arrangement of GRU elements is important for the glucocorticoid response

The effects of different GRU arrangements on glucocorticoid-dependent transcription were tested by transient transfections into FTO-2B hepatoma cells. Modified GRUs were placed upstream of the proximal enhancer and minimal promoter. After 24 h of induction with or without glucocorticoids, cell lysates were prepared. Luciferase values are presented as means ± S.E.M. for at least four experiments and the fold induction. The asterisk indicates significantly different results relative to parent construct a. The data show that inversion of the GRU (construct b) lowered the activity 2.5-fold, whereas inversion of the FoxA site only (construct c) decreased the activity 3-fold. Displacement of the C/EBP RE downstream of the FoxA- and GR-binding sites (construct d) decreased the activity 3-fold. Dex, dexamethasone.

Effects of variable spacer lengths between the C/EBP- and FoxA-binding sites in the GRU

A series of GRU constructs was made with deletions or insertions between the C/EBP and FoxA REs. These modified GRUs were placed upstream of the proximal enhancer and minimal promoter of the CPS gene, and tested by transient transfection into FTO-2B hepatoma cells. After 24 h of treatment with or without glucocorticoids, cells were lysed and luciferase values were measured. The results are expressed as means ± S.E.M. and fold induction from at least four experiments. Results that are significantly different from those with the parent construct c are indicated by asterisks. Shortening of the C/EBP–FoxA spacer (constructs a and b) significantly increased activity relative to the parent construct (c), whereas increasing the distance had no effect (constructs d–f). Dex, dexamethasone.

Optimization of the GRU REs increases activity and eliminates dependence on P3

When the CPS GRE was replaced by an optimized palindromic GRE, the C/EBP RE by a sequence selected for high-affinity binding [18] and the FoxA-binding site by a consensus sequence [19], glucocorticoid-induced GRU activity increased 1.4-fold (Figure 5, construct a). When the same optimizing modifications of the REs were introduced in the construct containing the inactivating P3 site mutation (Figure 5, construct d), GRU activity was increased 9-fold compared with the same construct carrying the native RE sequences, and became similar to that of the construct containing an intact P3 site and optimized REs (Figure 5, compare constructs a and d). Optimization of the REs can, therefore, also compensate for loss of P3. When the length of the FoxA–GR spacer exceeded 25 bp, optimization of the REs still increased GRU activity (Figure 5, construct e), but not to that of the P3-containing sequence. When we tested the effects of the optimized REs at FoxA–GR spacer lengths of 7 and 12 bp (Figure 5, constructs b and c), GRU activity was increased 1.8–1.9-fold with respect to the corresponding parent constructs, with preservation of the distance effects observed with the parent constructs. It thus seems that optimizing the GRU REs can compensate for loss of a functional

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Figure 4  Both the distance and the sequence of the region between the FoxA and GR REs influence the glucocorticoid response

A series of GRU constructs was made with deletions or insertions between the FoxA and GR REs. Modified GRUs were tested in conjunction with the proximal enhancer and the minimal promoter by transfection into FTO-2B hepatoma cells (A). The results are given as means ± S.E.M. for at least four experiments and the fold induction. The asterisks indicate that results are significantly different from those with construct e. The curved line indicates the helical expression profile with respect to the distance between the FoxA and GR REs. The triangles indicate the positions of the point mutations. (B) Sequences of the respective spacers. Italic characters represent nucleotides deviating from the parent sequence. Dex, dexamethasone.

A C/EBPβ RE can functionally substitute for the P3 RE

Since a 7 bp substitution in the middle of the P3-binding region completely inactivated the GRU (Figures 6A and 6D, construct c), we modified fewer nucleotides. On substituting 3 bp in the P3-binding region (Figures 6A and 6D, construct b), hormone-induced GRU activity decreased to 40% of that of the parent construct (construct a). In accordance, these modified P3 elements could not compete with the wild-type P3 probe (Figure 6B) for protein binding. To define the P3-binding region further, we used oligonucleotides spanning the P3-binding region in which all nucleotides left of the hexameric A-stretch in the centre of the P3-binding region, the three nucleotides directly left and right of
Specific mutations (indicated by the triangles) were introduced in the P3-binding region of the GRU to give P3 mutants and a construct in which the P3 RE was altered to a consensus C/EBP β RE (D). These GRUs were placed upstream of the proximal enhancer and minimal promoter of the CPS gene, and the constructs were transfected into FTO-2B hepatoma cells. After 24 h of induction with glucocorticoids, reporter-gene expression was measured in the cell lysates. Results are presented as mean ± S.E.M. luciferase values from at least four experiments and the fold induction (A). Asterisks indicate results that are significantly different from those with the parent construct a. (B) To prove specificity, the P3 mutants were also used in a competition assay. A radiolabelled P3-binding region was incubated with 3 µg of rat liver nuclear extract and separated on a 6 % (w/v) acrylamide gel (lane 2). Whereas addition of 0.1, 1 or 10 pmol of P3 oligonucleotide efficiently competed with the probe for protein binding (lanes 3–5), the same amounts of mutant P3 oligonucleotides could not (lanes 6–8 and 9–11). To define further the core P3-binding site, a similar competition assay was carried out (C): added to the reaction mixture was 0.1, 1 or 10 pmol of the parent P3 competitor (C, lanes 3–5; D, sequence a), or a competitor in which the left side (C, lanes 6–8; D, sequence e), middle (C, lanes 9–11; D, sequence f), or right side (C, lanes 12–14; D, sequence g) of the P3-binding region had been mutated. Dex, dexamethasone.

DISCUSSION

The present study shows that the functionally important elements of the CPS GRU are the binding sites for the GR and for the accessory proteins FoxA and C/EBP. The CPS GRU is, therefore, one of the simplest GRUs described. Although the experiments
Addition of C/EBP probes gave rise to a protein–DNA complex when incubated with nuclear extract (lanes 2 and 5).

To induce a supershift, C/EBP β sequence as probe. The probes were incubated with a C/EBP β protein–DNA complex (lane 6), but not the P3 protein–DNA complex (lane 3).

Figure 7 The P3-binding region is not a C/EBPβ-binding site

Supershift assays were performed using either the P3-binding region or the consensus C/EBPβ sequence as probe. The probes were incubated with a C/EBPβ-enriched COS-1 cell nuclear extract. To induce a supershift, C/EBPβ-specific antibodies were added to the samples. Both probes gave rise to a protein–DNA complex when incubated with nuclear extract (lanes 2 and 5). Addition of C/EBPβ-specific antibodies supershifted the C/EBPβ protein–DNA complex (lane 6), but not the P3 protein–DNA complex (lane 3).

Figure 8 Identification of the protein that interacts with the P3 site by Southwestern blot analysis

Crude and partially purified rat liver nuclear extracts were resolved on an SDS/9 %–PAGE gel and transferred to a PVDF membrane. Following renaturation of the proteins, the blot was incubated with radiolabelled P3 probe. The positions of the molecular size markers are indicated. The liver nuclear extract contained a protein migrating at 75 kDa that was able to bind the P3 sequence (lane 1). A P3-positive MonoS fraction contained the same protein (lane 3), whereas a P3-negative MonoS protein fraction lacked this P3 protein (lane 2).

also showed that the P3 site was essential for GRU activity in its wild-type configuration, we identified three modifications that rendered the GRU independent of P3. This finding suggests that P3 is not necessary to make an efficient GRU, but serves to facilitate the formation of an active DNA–protein complex on the CPS GRU. The finding that the CPS GRU loses a large part of its transactivating activity when placed in the inverse orientation before the CPS promoter (Figure 2, construct b) is at first glance incompatible with the definition of enhancers as regulatory regions that can activate transcription from a promoter independent of their orientation [20]. However, we did not observe a dependence on orientation when a larger fragment comprising the GRU was tested [21], which suggests that this dependence of GRU activity on its orientation when a larger fragment comprising the GRU becomes necessary to ensure activity of the GRU. P3 was, however, redundant at this spacer length when the REs in the CPS GRU were modified into high-affinity elements, but this modification was no longer effective at greater spacer lengths. These findings suggest that P3 functions to facilitate interaction between the FoxA and GRE REs at a distance where they have difficulty contacting each other. A strict requirement with respect to FoxA–GRE spacing is also observed in the PEPCK gene [24]. The distance of 18 bp that separates the FoxA-binding site (AF2) from GRE1 in this gene are critical for a full glucocorticoid response, with insertion of an additional helical turn decreasing expression levels.

Our results show that the CPS GRU is not maximally compact in the wild-type configuration. Instead, our data suggest that, by decreasing the C/EBP–FoxA spacer distance to 1 bp and the FoxA–GRE spacer distance to 2 bp (no P3), a unit would be generated that is very active, very responsive to glucocorticoids, very small and very simple, since it only comprises three REs. Clearly, a compact GRU does not need P3 for activity, since P3 is redundant at FoxA–GRE distances of 2 and 12 bp and when the other GRU REs were replaced by optimal REs. The question, therefore, emerges as to why P3 is integrated into this unit. Since the sequences of the GRU REs and the distance between the FoxA and GRE REs have evolved in such a way that it allows the CPS GRU to be regulated by P3, it is probable that proper regulation of the wild-type GRU requires the interplay of all four transcription factors. To fully understand P3 function, it is necessary to establish its identity. DNA-sequence analysis suggested that P3 could be a
C/EBPβ-binding site. When we tested this possibility by altering the P3 sequence into a consensus C/EBPβ site, we found a 3-fold increase in glucocorticoid-induced activity. However, using super-shift assays, we found that C/EBPβ could not bind the wild-type P3 region. Although P3 has resisted the elucidation of its identity so far, our Southwestern analysis indicates that it is a 75 kDa protein.

In conclusion, our results provide new insight into how GRUs work. GRUs are not just clusters of REs that are end-points of regulatory pathways. Instead, the sequence, orientation and spacing of the participating REs are all crucial parameters in the effectiveness of a GRU.

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