The hormonal regulation of carbamoylphosphate synthetase I expression
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The cyclicAMP-response unit in the distal enhancer of the rat carbamoylphosphate-synthetase gene is a bifunctional element

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**ABSTRACT**

The hepatic expression of the carbamoylphosphate-synthetase-I gene (CPS) involves a minimal promoter, an adjacent proximal enhancer, and a 469-basepairs distal enhancer at -6.3 kb. The 80-basepairs glucocorticoid-response unit (GRU) in the 3' half of the distal enhancer acts, in conjunction with a GRU in the proximal enhancer, as a strong activator of reporter-gene expression in response to glucocorticoids. Here, we report that the 5' half of the distal enhancer, which contains a cyclicAMP- (CRE), one FoxA-, and two C/EBP-response elements, and the binding site for the unidentified protein P1, functions as a 260-basepairs cyclicAMP-response unit (CRU) and is the sole mediator of the cyclicAMP response. South-western blot showed that four bands of $\geq 180$ kDa bind to the P1 sequence. Although a single GRU cannot confer glucocorticoid inducibility on the CPS promoter, it suffices to confer glucocorticoid inducibility on a construct containing a CRU, irrespective of whether the GRU is located proximal or distal relative to the promoter. The glucocorticoid induction, however, is highest when both GRUs are present. When reporter-gene expression is driven by both GRUs and the CRU, the GRUs no longer function as a unit. Instead, the glucocorticoid response only depends on the respective GREs in this configuration. We, therefore, refer to the CPS CRU as a bifunctional element, which mediates the cyclicAMP response as a genuine unit, whereas it acts as a glucocorticoid-accessory unit in the glucocorticoid response.
**INTRODUCTION**

Ammonia, a toxic product of amino-acid degradation, is detoxified in the liver by the urea cycle enzymes\(^1\). The expression of these urea cycle enzymes is confined to the periportal hepatocytes and can be modulated at the transcriptional level by hormones like glucagon, insulin, and glucocorticoids\(^2\). The carbamoylphosphate-synthetase-I gene (CPS; E.C. 6.3.4.16) is the rate-determining step of the urea cycle, converting ammonia, bicarbonate and ATP into carbamoylphosphate. CPS and ornithine transcarbamoylase are located in the mitochondrial matrix, whereas the last three urea-cycle enzymes reside in the cytosol.

The CPS regulatory regions for expression in liver comprise a 469-basepairs distal enhancer that is located 6.3 kb upstream of the transcription-start site, a proximal enhancer (-161 to -35 bp) and a minimal promoter (Figure 1, panel A;\(^3\)). The proximal enhancer features a GAGA-box and a glucocorticoid-response element (GRE), which, together, make up a proximal glucocorticoid-response unit (GRU)\(^4,5\). Transgenic mice carrying a reporter construct regulated by the distal enhancer, the proximal GRU and the minimal promoter show periportal expression in liver and responsiveness to glucocorticoids and cyclicAMP\(^3\). An 80-basepairs subregion in the 3' part of the distal enhancer was identified as a GRU. When combined with the proximal GRU, this distal GRU exerts about 50% of the glucocorticoid-induced activity relative to the complete distal enhancer. Footprinting analysis of the distal GRU has revealed the presence of binding sites for the ubiquitously expressed glucocorticoid receptor (GR), the liver-enriched transcription factors CCAAT/enhancer-binding protein (C/EBP) and forkhead box A (FoxA/HNF3), and a binding site for an unknown protein denoted as P3\(^6\). We previously showed that neither the distal nor the proximal GRU can mediate a glucocorticoid response alone. Instead, both units need to cooperate to mediate glucocorticoid-dependent transcription\(^5\).

Our previous research\(^6\) assigned the effects of glucocorticoids on transcription exclusively to both GRUs. However, the 16-fold reduction in basal expression and the complete absence of cyclicAMP responsiveness when the distal GRU instead of the complete distal enhancer controlled reporter gene expression, suggested a role for additional factors in the distal enhancer that are lacking in the distal GRU. It is not uncommon that another set of transcription factors is required to elicit a response to a different hormone. In the PEPCK gene, for example, the glucocorticoid and cyclicAMP response each require a different array of transcription factors, acting in concert to mediate transcription\(^7\). The 5' half of the PEPCK
enhancer in combination with a downstream CRE mediates the glucocorticoid response, whereas the 3' half in combination with this same downstream CRE mediates the cyclicAMP response. We, therefore, tested the hypothesis that the 5' region of the distal enhancer, which contains a cyclicAMP-responsive element (CRE), two C/EBP- and one FoxA-response elements, and the binding site for an unidentified protein, P1, functions as a cyclicAMP-responsive unit (CRU) in the CPS gene. Here, we show that this part of the enhancer indeed functions as a unit and is the sole mediator of the cyclicAMP response. In addition, this CRU can participate in establishing a glucocorticoid response from otherwise silent proximal or distal GRUs and, thus, functions as a glucocorticoid-accessory unit.

METHODS

Tissue culture

FTO-2B rat hepatoma cells were grown in DMEM/F12 medium (Invitrogen life technologies, Carlsbad, CA, USA) supplemented with 10% foetal calf serum in a 5% CO$_2$ atmosphere at 37°C. Because FTO-2B cells are deficient for the repressing protein kinase A Rlα subunit, they have constitutive protein kinase A activity.

Oligonucleotides and plasmids

All enhancer-promoter constructs were cloned into the pSPluc+ vector (Promega, Madison, WI, USA), with the bovine growth-hormone polyA tail between the XbaI-EcoRV sites downstream of the luciferase gene. The minimal CPS promoter with or without the proximal CPS enhancer (-161 to -35) was inserted into the KpnI-HindIII sites of the polylinker. Enhancer regions with or without mutations were cloned into the BamHI and PstI sites directly upstream of the minimal promoter or proximal enhancer when present. The enhancer regions were generated by PCR using (modified) oligonucleotides purchased from Invitrogen life technologies. The nucleotide sequence in these modified enhancers was verified using the Big dye terminator kit (Amersham Biotechnologies, Little Chalfont, UK).

Transfections

The activity of enhancer-promoter reporter constructs was assessed by electrotransfection of 1·10$^7$ FTO-2B hepatoma cells with 20 μg of tester plasmid DNA and 2 μg pRL-CMV control DNA (Promega). Transfected cells were split into four equal parts and cultured in four 2 cm$^2$ wells. 24 hours posttransfection the medium was replaced and cultured for another 24 hours; one well was left untreated, whereas
the other wells were supplemented with 100 nM dexamethasone (Centrafarm, Etten-Leur, The Netherlands), 250 μM chlorophenylthio-cyclicAMP (Sigma-Aldrich Corp., St Louis, MO, USA), or both. 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 variation. Transfection results are presented as means of at least four experiments and their SEM. To analyse the differences between constructs, the non-parametric Kruskal-Wallis test was employed. Results that were considered significantly different from the control (p<0.05) are marked by an asterisk. Unless indicated otherwise, the distal enhancer in conjunction with the proximal enhancer and the minimal promoter functions as control.

South-western blotting
The proteins that interact with the P1 and P2 regions in the distal enhancer were provisionally characterised by south-western blotting. 25 μg rat liver nuclear extract, prepared according to the method of Sierra, was mixed with 2x sample buffer (25% 0.5 M Tris-HCl, pH 6.8, 4% SDS, 0.1% bromophenol blue, 20% glycerol, and 5% β-mercaptoethanol) and separated on a 9% SDS-PAGE minigel in Laemmli buffer (50 mM Tris base, 384 mM glycine, 1% SDS, pH 8.3) at 50 volts until the bromophenol blue migrated off the gel. After 1 hour preincubation in blotting buffer at 4°C (25 mM Tris base, 192 mM glycine, pH 8.3) the proteins were electroblotted onto Immobilon-PVDF membrane (Millipore, Billerica, MA, USA) for three hours at 50 volts. The blot was incubated for 15 minutes in binding buffer (20 mM HEPES pH 7.9, 5 mM MgCl₂, 50 mM NaCl, 1 mM DTT) to allow the proteins to renature. After blocking the membrane for 1 hour in blocking buffer (5% nonfat dried milkpowder, 0.01% Tween-20 in binding buffer), the blot was incubated overnight with the radiolabeled double-stranded probe at a concentration of 1×10⁶ cpm per ml in hybridisation buffer (0.25% nonfat dried milkpowder, 0.01% Tween-20 in binding buffer). Following two washing steps of 20 minutes with hybridisation buffer and one washing step in binding buffer, the blot was dried and exposed overnight to phosphorus screen and analysed using a Storm 860 PhosphorImager apparatus (Molecular Dynamics, Sunnyvale, CA, USA).
RESULTS

The 5' region of the distal enhancer harbours the cyclicAMP response

Transient transfection of FTO-2B hepatoma cells showed that the minimal promoter alone (Figure 1, construct a) and the combination of the minimal promoter with either the proximal (construct b) or the distal GRU (construct c) did not exhibit any activity per se. The combination of all three elements (construct d) was very effective in inducing glucocorticoid-dependent gene expression. However, this entire distal enhancer was twice as effective as the distal GRU alone upon exposure to glucocorticoids (compare constructs d and e).

Figure 1. The cyclicAMP response is mediated by the 5' region of the distal enhancer. FTO-2B hepatoma cells were transiently transfected with CPS enhancer-promoter construct. Following 24 hours induction with glucocorticoids and/or cyclicAMP, cells were harvested and luciferase values were measured. Asterisks indicate significantly different results relative to construct e. The results show that the minimal promoter (a), the proximal GRU (b), and distal GRU do not contain any activity per se. Only the combination of these regions renders a glucocorticoid response (d). When linked to the proximal GRU, the complete distal enhancer has (e) a twofold higher glucocorticoid response than the distal GRU. In addition, the complete distal enhancer is also responsive to cyclicAMP.
Moreover, basal activity and the activity in response to cyclicAMP were virtually nil in conjunction with the distal GRU, but rose to substantial levels when the entire distal enhancer was present (compare constructs d and e). These discrepancies suggested that elements in the 5' half of the distal enhancer contributed to basal activity and the cyclicAMP response.

The 5' region of the distal enhancer functions as a unit
Since the 5' distal enhancer region contains a cluster of transcription factor-binding sites, we tested the hypothesis that this region acts as a unit in activating transcription. When the region corresponding to the distal GRU was removed (Figure 2, constructs a and b), basal activity and glucocorticoid inducibility of reporter-gene expression was more than 2-fold reduced, whereas cyclicAMP responsiveness remained intact.

Figure 2. The 5' region of the distal enhancer harbours a CRU. Distal enhancer regions were placed upstream of the proximal GRU and minimal promoter in a luciferase-reporter construct and were transfected to FTO-2B hepatoma cells. After 24 hours induction with glucocorticoids and/or cyclicAMP, cells were harvested and luciferase activity were measured. Significantly different results relative to the complete distal enhancer (construct a) are indicated by an asterisks. Removal of the GRU from the distal enhancer (b) decreases the glucocorticoid response but fully retains the cyclicAMP response. When the P2 element was deleted (construct c) the glucocorticoid response was restored to the same level as the complete distal enhancer, while the cyclicAMP response remained unchanged. Deletion of the C/EBP(ii) (d) or P1 (e) strongly reduced both the cyclicAMP and the glucocorticoid induced activity, thereby marking the 5' and 3' boundaries of the CRU. Mutation of the CRE within the distal enhancer abolishes the cyclicAMP response (f).
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When the region covering the P2 footprint was additionally removed (Figure 2, construct c), both basal and hormone-induced activity were similar to that observed with the complete distal enhancer (Figure 2, construct a). Further trimming at the 3' end (construct d) rendered the construct much less active, without changing the fold-induction by hormones. Removing the region corresponding to the P1 footprint at the 5' end of the distal enhancer (construct e) also reduced reporter-gene expression without affecting hormonal inducibility. Mutation of the CRE within the complete distal enhancer similarly reduced reporter-gene activity, but also abrogated the response to cyclicAMP, whereas the responsiveness to glucocorticoids was retained (construct f). Together, these results show that the transcription factors that bind to the 5' half of the distal enhancer function as a cyclicAMP-response unit (CRU).

The elements of the distal GRU do not act as a unit in the context of the distal enhancer when the proximal GRU is present

Figure 2 showed that the CRU and the complete distal enhancer mediate a similar response when combined with the proximal GRU, which raises the question of the function of the distal GRU. We, therefore, mutated the respective response elements within the distal GRU in the context of the entire distal enhancer. None of the mutations in the accessory elements of the GRU (C/EBP(iii), FoxA(ii), P3) had an effect on reporter-gene expression or fold-induction (Figure 3, constructs b, c, d), except that of the GRE, which caused a 60% decrease in activity in response to glucocorticoids without affecting the cyclicAMP response (construct e). The composing elements of the distal GRU, therefore, do not act as a unit in the context of the entire distal enhancer and proximal GRU, but its GRE does influence glucocorticoid inducibility.
Identification of a bifunctional unit

Figure 3. In the context of the 469-basepairs enhancer, the distal GRU does not act as a unit. The distal GRU REs were mutated in the context of the entire 469-basepairs distal enhancer. These DNA constructs were placed upstream of the minimal promoter and proximal enhancer, and were transfected to FTO-2B hepatoma cells. Following 24 hours induction with glucocorticoids and/or cyclicAMP, cells were harvested and luciferase values were measured. The results show that mutating any distal GRU-accessory element does not significantly influence reporter-gene activity (b, c, d). Only mutation of the GRE in the distal enhancer (e) or in the proximal enhancer (f) reduces the glucocorticoid response relative to the parent construct (a).

A single GRU suffices to confer glucocorticoid inducibility in the presence of a CRU

Figure 1 demonstrated that the glucocorticoid response requires the presence of both the distal and the proximal GRUs. We, therefore, tested whether the proximal GRU was also necessary for cyclicAMP responsiveness. Figure 3 (construct f) shows that when the complete distal enhancer was present, mutation of the GRE in the proximal GRU reduced glucocorticoid inducibility and reporter-gene expression to a similar extent as when the GRE in the distal GRU was mutated (construct e), demonstrating that the quantitative contribution of both GREs to the glucocorticoid response of the CPS promoter/enhancer is similar. If instead of the distal enhancer, only the distal GRU had been present, mutation of the proximal GRE would have silenced the promoter 5. As in the case of the GRU in the distal enhancer, the GRU in the proximal enhancer therefore no longer functions as a unit when a CRU is present. Figure 4 shows that cyclicAMP inducibility remained intact when the proximal GRU was deleted in its entirety (compare construct b with construct a). When neither the distal nor the proximal GRU were present (Figure 4, construct c), cyclicAMP inducibility
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Figure 4. The CRU is sufficient to mediate the cyclicAMP response from the minimal promoter. Enhancer-promoter constructs were transiently transfected to FTO-2B hepatoma cells. After 24 hours induction with glucocorticoids and/or cyclicAMP, luciferase values were measured. Results that are significantly different from the complete distal enhancer in conjunction with the proximal GRU (construct a) are indicated by an asterisk. The data show that the complete distal enhancer is still able of mediating both a glucocorticoid and a cyclicAMP response in absence of the proximal GRU although the glucocorticoid-induced activity has strongly decreased (b). Without proximal GRU, the CRU alone can fully mediate cyclicAMP-dependent transcription from the minimal promoter, but the glucocorticoid response has all but vanished (c).

remained intact, but glucocorticoid inducibility had virtually disappeared and reporter-gene activity was severely reduced. These findings demonstrate that the CRU is the sole determinant of the cyclicAMP response, whereas the presence of either the distal (Figure 4, construct b) or the proximal GRU (Figure 2, construct c) is necessary for glucocorticoid responsiveness, even though these GRUs are inactive when they are tested without the CRU (Figure 1, constructs b and c). These observations indicate that strong glucocorticoid responsiveness requires either the presence of two GRUs or that of a GRU and a CRU. Since the CRU alone cannot confer glucocorticoid responsiveness (Figure 4, construct c), the single remaining GRU is apparently as potent in this context as when 2 GRUs, but no CRU is present (Figure 1, construct d).

P2 and FoxA(iii), two inhibitory elements in the distal enhancer
The deletion of the distal GRU from the distal enhancer reduced glucocorticoid inducibility and level of reporter-gene expression 3-4-fold, while hardly affecting cyclicAMP inducibility (Figure 5, construct a and c). Since the additional deletion of the P2 element completely restored glucocorticoid inducibility and level of reporter-gene expression (Figure 2, construct c), we mutated this element in the context of the intact upstream enhancer. This mutation did not significantly change reporter-gene
Identification of a bifunctional unit

Figure 5. P2 and FoxA(iii) repress reporter-gene expression. Distal enhancer regions were placed upstream of the proximal GRU and minimal promoter in luciferase-reporter constructs. The DNA constructs were transiently transfected to FTO-2B hepatoma cells and cultured for 24 hours in presence of glucocorticoids and/or cyclicAMP. The results show that deletion of the FoxA(iii) RE significantly increases expression and inducibility (b) relative to the complete distal enhancer (a). Removal of the distal GRU from the distal enhancer shows a reduction of the glucocorticoid responsiveness while the cyclicAMP responsiveness is maintained (c). Mutation of P2 in the distal enhancer does not significantly increase reporter-gene activity and inducibility (d) relative to its parent construct (a).

activity (Figure 5, compare constructs a and d), but due to a decreased basal activity, it increased the response to both glucocorticoids and cyclicAMP 1.5-fold. The presence of P2, therefore, mainly interferes with glucocorticoid responsiveness of the CRU in conjunction with the proximal GRU.

At the most 3' end of the distal enhancer, a third FoxA element is located (FoxA(iii)) that matches a consensus FoxA sequence well. Since it does not belong to either the CRU or distal GRU, we questioned its role in the function of the distal enhancer. Deletion of this FoxA site from the distal enhancer resulted in a 50% increase in glucocorticoid and cyclicAMP inducibility and in the level of expression of the reporter gene (Figure 5, construct b). Both this observation and that on P2 suggest that the binding of a protein between the distal and proximal GRU inhibits a productive interaction between these units.

The P1 and P2 REs bind proteins of > 180 and 65 kDa, respectively
To preliminarily characterise the proteins that interact with the P1 and P2 regions, South-western blots were prepared with a rat-liver nuclear extract that EMSA experiments had shown to contain the interacting proteins (data not shown) and
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radiolabeled probes corresponding to the P1 and P2 regions. For P1, we identified four bands of 180 kDa and larger (Figure 6). When we used the P2 probe, a very strong band of ~65 kDa was visualised (Figure 6). When we compared these data with those of candidate transcription factors in a transcription-factor database, no convincing matches were found.

![Liver nuclear extract](image)

**Figure 6. P1 and P2 bind proteins of > 180 and 65 kDa respectively.** South-western blots were prepared using rat liver nuclear extract. Radiolabeled double-stranded probes (P1: tggtttatattatcacatatctgtt; P2: gtatgtctgtgacatgaccc) were incubated with these blots. Four bands of 180 kDa and larger were identified using the P1 probe, whereas a very strong band at 65 kDa was observed when using the P2 region as probe.

**DISCUSSION**

The CRU is the sole mediator of the cyclicAMP response

Our previous research identified regulatory elements for CREB, FoxA and C/EBP, as well as binding sites for the unknown proteins P1 and P2 in the 5' region of the distal enhancer of the CPS gene \(^6\). Although CREB binds preferentially to a symmetrical CRE (TGACGTCA), it can also activate genes that contain a CRE half-site (CGTCA) such as PEPCK \(^14\), TAT \(^15\), CPS \(^6\), angiotensin \(^16\), and the P4 promoter of minute virus \(^17\). We now showed that P1 and P2 interact with proteins of > 180 kDa and 65 kDa, respectively. All these elements, except P2, function as a unit that mediates the cyclicAMP responsiveness of the distal CPS enhancer, since deletion of P2 increases the hormonal responses, whereas deletion of the other elements decreases the response. We, therefore, refer to it as a cyclicAMP-responsive unit (CRU).

Since mutation of the CRE in the context of the entire distal enhancer showed no cyclicAMP response, the presence of a CRE in the CPS distal enhancer is a strict requirement for responsiveness to cyclicAMP. In contrast, the CRU is able of
participating in the glucocorticoid response without the presence of a GRE within this unit. Glucocorticoids have been reported to activate transcription in absence of a GRE. The upregulation of C/EBPβ by glucocorticoids appears to mediate the glucocorticoid response of the arginase gene\textsuperscript{18} and may be cited as an example of an indirect regulation of transcription by glucocorticoids. However, the glucocorticoid responsiveness of the CPS CRU requires the presence of at least one GRE within the expression construct, showing that in this gene, glucocorticoids directly modulate transcription.

The higher basal activity of the construct containing the CRU and the proximal GRU as compared to the construct containing the distal and proximal GRUs probably reflects the constitutive activity of protein kinase A in FTO-2B cells\textsuperscript{10}. As a result, a fraction of cellular CREB is already CRE-bound in absence of cyclicAMP. Nevertheless, CREB phosphorylation by protein kinase A is still a rate-limiting step, since addition of cyclicAMP results in a further increase in activity up to 3-fold.

**The GRUs do not act as units in the context of a CRU**

Inclusion of the distal and/or proximal GRU in an expression construct does not increase cyclicAMP responsiveness of the CRU, underscoring our earlier conclusion that the presence of a CRU is the only requirement for a productive cyclicAMP response. A single GRU cannot confer glucocorticoid inducibility on the CPS promoter\textsuperscript{5}, but suffices to confer glucocorticoid inducibility on a construct containing a CRU, irrespective of whether this GRU is located proximal or distal relative to the promoter. We previously showed that the presence and proper alignment of all REs in both the distal\textsuperscript{19} and proximal GRU\textsuperscript{5} are required for a glucocorticoid-dependent transcription from the minimal promoter in the absence of a CRU. When together, the distal and the proximal GRU thus truly act as transcriptional units. However, when the CRU is present, only mutation of the GRE decreases the glucocorticoid responsiveness of the expression constructs. In the context of a CRU, the proximal and distal GRUs therefore no longer function as transcription units. Instead, their GREs form functional units with the CRU, each contributing to a similar extent to the overall response. One could therefore say that the CRU acts as an accessory unit in mediating the glucocorticoid response. At first glance, such a finding appears paradoxical. We, therefore, hypothesise that conditions exist in which the CPS CRU is selectively silenced. As far as we are aware, such a pronounced modular behaviour of hormone-dependent transcriptional units has not yet been reported.
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Determinants of cyclicAMP- and glucocorticoid-dependent expression of CPS

In summary, our study shows that the CRU in the distal CPS enhancer is necessary and sufficient for all cyclicAMP-dependent transcriptional activity mediated by the distal enhancer. In addition, this CRU can team up with GREs in the distal and proximal enhancer to mount a strong glucocorticoid response. We therefore refer to the CRU as a bifunctional unit, which mediates the cyclicAMP response as a genuine unit, whereas it acts as a glucocorticoid-accessory unit in the glucocorticoid response. In the absence of a functional CRU, however, the GREs in the distal and proximal enhancers team up with adjacent accessory elements to form genuine GRUs that are not responsive to cyclic AMP.
REFERENCES


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