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Chapter 1

Mechanisms of glucocorticoid signalling

Onard J.L.M. Schoneveld, Ingrid C. Gaemers, Wouter H. Lamers
Chapter 1

**ABSTRACT**

It has become increasingly clear that glucocorticoid signalling not only comprises the binding of glucocorticoid receptor (GR) to its response element, but also involves complex interactions between different signalling pathways. Glucocorticoid action can result in negative regulation by GR-binding to negative glucocorticoid-response elements (nGREs) or via protein-protein interactions. Positive regulation of glucocorticoid-responsive genes can be the result of GR binding to GREs (primary response) or due to upregulation of transcription factors that bind to the regulatory elements of such genes (secondary response). To establish a primary response, GR can bind to simple GREs, or to GREs and GRE half sites (GRE½s) that are part of a regulatory unit. A response unit allows a higher level of glucocorticoid induction than simple GREs and, in addition, allows the integration of tissue-specific information with the glucocorticoid response. Presumably, the complexity of such a glucocorticoid-response unit (GRU) depends on the number of pathways that integrate at this unit. When all pathways that converge into a GRU are activated, the GRU can mediate the glucocorticoid response onto the transcription-initiation complex. Because GRUs are often located at distant sites relative to the transcription-start site, the GRU has to find a way to communicate with the basal-transcription machinery. We propose that the activating signal of a distal enhancer can be relayed onto the transcription-initiation complex by coupling elements located proximal to the promoter.
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I. SCOPE

Glucocorticoid hormones regulate numerous physiological processes, such as regulation of glucose, fat and protein metabolism, and anti-inflammatory and immuno-suppressive actions. The classical model for glucocorticoid function is based on binding of a hormone-bound glucocorticoid receptor (GR) dimer to glucocorticoid-response elements (GREs) in the promoters of target genes, thereby changing the expression of these genes. GRs are expressed in most tissues, but regulate a set of target genes that differs in each tissue. This cell-dependent regulation invoked revision of the classical model and led to the discovery of cross-talk between different transcription factors over a decade ago.

This extended model of glucocorticoid regulation is nicely illustrated in the liver, where glucocorticoids enhance the transcription of genes involved in gluconeogenesis, amino-acid catabolism and the acute-phase inflammatory response. The expression of these genes is influenced by glucocorticoids in a direct way through binding of the GRs to GREs present in the promoters of these genes. In addition, these GREs are part of composite regulatory elements or GRUs (glucocorticoid-response units), composed of binding sites for several transcription factors, which exhibit a remarkable modular similarity between the gluconeogenic genes.

In the promoters of the above mentioned gluconeogenic genes the GREs consist of the classical palindromic GR-binding sequence to which a hormone-bound GR dimer can bind. However, in recent years a number of genes have been described where a GRE half-site (GRE½s), i.e. only one half of the classical palindrome, in the promoter is sufficient to relay glucocorticoid signalling. Similarly, binding of GR monomers has also been described. In addition to the usual direct, positive effect of GRs on gene expression, there are also a number of examples where glucocorticoids exert a negative effect on gene expression or where glucocorticoids act in an indirect way.

Despite our extended comprehension of glucocorticoid signalling, the intriguing question what determines cell- and promoter-dependent glucocorticoid signalling still stands and it seems that another revision of the model for glucocorticoid regulation of (tissue-specific) gene expression is required. To address this point, we discuss in this review the composition of the GRUs present in the promoters of gluconeogenic genes, the transcription factors that bind to these GRUs, the interactions between these factors, and other factors that may influence the activity of GRUs, such as higher order chromatin structure and nuclear organisation.
To our knowledge, no earlier publications have extensively reviewed the composition of regulatory regions in glucocorticoid-regulated genes. We also outline the present state of knowledge about glucocorticoid signalling as outlined above and envision how all this may lead to cell- and promoter-specific regulation.

II. ROLE OF GLUCOCORTICOID SIGNALLING IN THE EXPRESSION OF CATABOLIC ENZYMES

Glucocorticoids, named after their role in maintaining glucose homeostasis, are produced in the adrenal cortex. Responses to glucocorticoids are mediated by the glucocorticoid receptor that is being translocated to the nucleus upon glucocorticoid binding and can there regulate the expression of many genes by complex regulatory mechanisms (see later section). Glucocorticoids exert a myriad of responses in different mammalian cells. They can up- and down-regulate a wide variety of genes affecting a number of critical metabolic and inflammatory pathways. Increased glucocorticoid levels adapt metabolic pathways to meet altered energy demands. To provide glucose, the rate of gluconeogenesis, lipolysis, and proteolysis are increased.

Figure 1. The amino-acid degrading enzymes, the urea cycle, and the citric-acid cycle are closely linked in gluconeogenesis. To provide glucose, amino acids are degraded enzymatically. The resulting carbon skeletons enter the gluconeogenic pathway or are oxidised in the citric-acid cycle, while ammonia is detoxified by the urea cycle enzymes.
Gluconeogenesis from amino acids generates the toxic metabolite ammonia. Coincident with the conversion of the carbon skeletons of the amino acids into glucose, the ammonia resulting from deamination of the α-amino group of amino acids is detoxified by the ornithine (urea) cycle in the liver (Figure 1). The regulation of the expression of urea cycle and gluconeogenetic enzymes is, perhaps for that reason, closely linked. Studies of glucocorticoid signalling in catabolic pathways have unravelled many aspects of the molecular mechanism underlying the regulation of catabolic genes.

### III. LIGANDS FOR THE GLUCOCORTICOID RECEPTOR

Besides regulation of catabolic processes, glucocorticoids inhibit a number of inflammatory responses. Therefore, glucocorticoids are often used as anti-inflammatory therapeutic agents. Long-term treatment with high levels of glucocorticoids unfortunately also upregulates the expression of catabolic genes, leading to a number of severe side effects amongst which are fat redistribution, weight gain, hyperglycemia, and osteoporosis.

Inflammatory responses act via pro-inflammatory transcription factors such as nuclear factor κB (NF-κB) and activator protein-1 (AP-1) by direct binding to their response elements in target genes. Mice harbouring a mutation in the dimerisation domain of the glucocorticoid receptor, which was considered to jeopardise the interaction with DNA, repress AP-1-dependent collagenase expression as efficiently as animals with the wild type receptor. These and other results suggested that the repressive effect of GR on the inflammatory response is independent of DNA-binding. Although the mechanism is still unknown, several models exist to explain the glucocorticoid-mediated repression of AP-1 and NF-κB signalling. One of them involves competition between GR and AP-1 for CBP, which is present in the cell in limiting amounts. However, the glucocorticoid-dependent repression of both AP-1- and NF-κB-mediated gene expression is independent of the amount of CBP in the cell. Furthermore, the GR-mediated repression of transactivation by the p65 subunit of NF-κB is increased in the presence of CBP, suggesting that CBP functions as an integrator of the NF-κB-GR cross talk. Another model suggested upregulation of I-κB, the inhibitor of NF-κB action, in some tissues in response to glucocorticoids, but a GR-mutant that was unable to upregulate I-κB, still repressed an NF-κB regulated gene. However, recent results showing that the dimerisation-impaired GR can bind DNA and can upregulate GRE-dependent
genes\textsuperscript{16} have shed some doubts on whether the anti-inflammatory actions of glucocorticoid are indeed independent of DNA-binding.

Ligands that can uncouple the transactivating from the transrepressing properties of GR can potentially reduce the adverse side effects of glucocorticoid action\textsuperscript{17}. Such ligands would have to bring about a different response depending on their ability to induce a specific conformation on the receptor and, hence, a conformation-dependent interaction with transcription factors, co-activators and components of the basal transcription machinery\textsuperscript{18}. Besides the naturally occurring glucocorticoids cortisol, cortisone, and corticosterone\textsuperscript{19} artificial glucocorticoids like dexamethasone, prednisolone, and clobetasol exist that differ in their ability to induce GRE-dependent gene expression and trans-repress the pro-inflammatory transcription factor nuclear factor \( \kappa \)B (NF-\( \kappa \)B). However, glucocorticoids with increased anti-inflammatory effects have concomitantly increased GRE-dependent gene activation, and thus also stronger side effects. Clobetasol, for instance, strongly represses NF-\( \kappa \)B-dependent transcription, but has also a high capacity to induce GRE-dependent transcription, whereas hydrocortisone is less effective in repressing NF-\( \kappa \)B-dependent transcription and in activating GRE-dependent transcription\textsuperscript{20}. The artificial glucocorticoid RU 24858 appeared to be more promising in this respect, as it was shown to transrepress AP-1 in vitro, while having a limited effect on GRE-based reporter genes\textsuperscript{21}. However, when this glucocorticoid was tested in vivo, it was found to have similar anti-inflammatory activity and side effects as other glucocorticoids. Despite all these studies, therefore, the discovery of artificial glucocorticoids with an improved therapeutic ratio has had limited success thus far.

IV. GLUCOCORTICOID HORMONE RECEPTOR

1. Protein structure
The glucocorticoid receptor belongs, together with the androgen receptor, thyroid receptor, mineralocorticoid receptor, and estrogen receptor to the family of steroid receptors and is ubiquitously expressed. Like the other steroid nuclear receptors, the glucocorticoid receptor is a modular protein with distinct functional and structural domains, comprising a DNA-binding domain, a ligand-binding domain and two transcription-activation functions (AF1 and AF2). GR mRNA levels are highest in lung, spleen, brain, and liver. Expression is upregulated in adrenalectomised rats and downregulated by dexamethasone\textsuperscript{22}. Although only one gene has been identified, several GR isoforms exist as a result of alternative splicing and the use of multiple promoters\textsuperscript{23}. Nuclear import of GR\( \alpha \) (777 amino acids), the main GR isoform, seems
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to be repressed by its ligand-binding domain. Only upon ligand binding, this repressive effect is released allowing nuclear import. Hormone binding, transactivation, dimerisation, and nuclear localisation of GRα is determined by 12th helix of the ligand-binding domain that harbours the transactivation function AF-2 (see later section) 24. In GRβ (742 amino acids), this stretch of 50 amino acids is replaced by a unique 15-amino acids tail that keeps the protein constitutively localised in the nucleus. The physiological role of GRβ is still a matter of debate. It has become clear that GRβ does not possess any transactivating activity. Some groups, however, have reported that GRβ can act as a dominant negative repressor due to dimerisation with GRα 25,26, whereas others found no significant repressive activity 27. In humans, a third isoform, GRγ has been identified that differs from the main isoform, GRα, by the presence of an additional amino acid in the DNA-binding domain as the result of alternative splicing 28.

DNA-binding domain
The DNA-binding domain (DBD) contains the ability to bind DNA. The "P-box" within this region contains the amino acids that are required for the specific recognition of the response element. Amino acids in the DNA-binding domain of the rat glucocorticoid receptor interact with specific nucleic acids in the major groove of the DNA helix 29. A "D-box" in the DNA-binding domain is involved in the dimerisation of the protein. Although monomeric in solution, the DNA-binding domain forms a dimer upon interacting with its response element. The DNA-binding domain cannot dimerise itself, but interaction with a GRE acts as an allosteric activator by providing a scaffold to bind the subunits in the correct position for dimerisation. Thus, binding of the first subunit favours binding of the second 30.

Ligand-binding domain
Recently, the crystal structure of the ligand-binding domain of hGRα was resolved 31,32. The ligand-binding domain is composed of α-helices and β-sheets that form a hydrophobic pocket and an additional side pocket. This side pocket is thought to allow the selective binding of glucocorticoids and some mineralocorticoids that have larger substituents at the C17α position than other steroids 33. Several models suggest that upon ligand binding, the ligand-binding domain undergoes a conformational change thereby 'closing' the pocket. Interestingly when single amino acids in the ligand-binding domain are mutated, the binding affinity changes and as may the specificity for different ligands. When parts of the ligand-binding domain are
deleted, ligand binding is reduced. In contrast, deletion of most of the ligand-binding domain (leaving amino acids 1 to 550) results in a constitutively active receptor. These data therefore suggest that the ligand-binding domain may function as a repressor of the receptor that can be de-repressed by ligand binding.

The interaction between co-activators and GR is very sensitive to the structure of the ligand that is bound in the pocket. The conformational sensitivity of co-activator interaction with GR is due to the changes in receptor structure brought about by the structure of the ligand bound in the pocket of the ligand-binding domain. It has been proposed that the receptor condenses around the ligand during the binding reaction, thereby transmitting structural changes in the ligand directly to the receptor and to the receptor surfaces that interact with co-activators. Thus, ligands may change the ability of the receptor to bind to co-activators.

Transactivation domains
The N-terminal part of the glucocorticoid receptor contains the glucocorticoid-independent AF1 transactivation domain. This region has been shown to interact with the TFIID complex and TBP of the general transcription machinery. It has been suggested that under specific conditions, this region forms an α-helical conformation that is essential for transactivation.

The second transactivation domain, AF-2, is contained within the LBD at the C-terminal side of GR. In contrast to AF-1, transactivation by AF-2 is dependent on ligand binding. AF-1 and AF-2 can interact with certain coactivators (see later section).

2. Signalling cascade
The glucocorticoid receptor is expressed in many cell types at a density of 2,000 to 30,000 per cell. In its unliganded state, most of the glucocorticoid receptor is located in the cytoplasm. Interactions with a chaperonine complex hold the receptor in an inactive state poised to bind its ligand. This complex comprises the heat-shock proteins hsp40, hsp70, hsp90, and in addition p23 and p60. It is postulated that hsp70 initiates the opening of the hydrophobic steroid-binding pocket in an ATP-dependent manner such that hsp90 can associate with the ligand-binding domain (LBD) of the receptor keeping the receptor in a ligand-receptive state.

In its unliganded state, the receptor is phosphorylated and becomes hyperphosphorylated upon ligand binding. Mouse GR contains seven phosphorylation sites that are located in the N-terminal region of the protein. In the
absence of hormones, GR is predominantly phosphorylated at Ser\textsuperscript{203}, but addition of hormones increases phosphorylation at both Ser\textsuperscript{203} and Ser\textsuperscript{211}. Phosphorylation at Ser\textsuperscript{211} coincides with increased transactivation properties of GR, possibly reflecting a conformational change that modulates the interaction with co-activators.\textsuperscript{46,47} The compartmental localisation of GR coincides with an altered phosphorylation status of GR: GR phosphorylated at Ser\textsuperscript{203} localised predominantly to the cytoplasmic compartment, whereas phosphorylation at Ser\textsuperscript{211} localised the receptor to both the cytoplasmic and the nuclear compartment. The double-phosphorylated GR, which is the result of glucocorticoid treatment, acts as a trigger to specifically dephosphorylate the receptor at Ser\textsuperscript{203} resulting in the mono-phosphorylated form that accumulates in the nucleus. When leaving the nuclear compartment, GR becomes also dephosphorylated at Ser\textsuperscript{211}. At this point, GR can either be recycled by recruitment in a chaperonin complex, which facilitates Ser\textsuperscript{203} phosphorylation, or be degraded.\textsuperscript{46}

The glucocorticoid response can be divided into two types. In the primary response, activated glucocorticoid receptor directly activates the transcription of target genes by interacting with their regulatory regions. Two mechanisms have been described to mediate a primary response (regulation via a GRE). The liganded receptor can interact with (general) transcription factors or co-activators to directly trans-activate gene expression. GR can, for instance, interact with RNA polymerase, for which a set of co-activators are utilised to bridge the gap between GR itself and the polymerase.\textsuperscript{48} A second mechanism involves the recruitment of co-activators with chromatin-remodelling activity to the GRE. The local chromatin modification can facilitate or obstruct binding of other transcription factors that are involved in transactivation. In the tyrosine aminotransferase (TAT) gene, GR binds the GRU first, inducing rearrangement of the chromatin such that other factors can bind and subsequently activate transcription.\textsuperscript{49}

The secondary response requires de novo synthesis of proteins (e.g. transcription factors and co-activators) that are upregulated by glucocorticoids, which can in turn activate transcription of target genes. The arginase gene that lacks a GRE, for instance, relies on this secondary response for its glucocorticoid-dependent induction of gene expression. In this gene, the upregulation of C/EBP\(\beta\) by glucocorticoids mediates the glucocorticoid-response.\textsuperscript{50}

Coactivators form a class of transcriptional regulators that do not bind DNA. Instead, these proteins exert their effect by interacting with transcription factors and/or components of the basal transcription machinery, or by altering local chromatin structure. The coactivators capable of interacting with the glucocorticoid
receptor comprise members of the p160 family of coactivators, CBP/p300, and p/CAF. Amongst the p160 family members are the glucocorticoid receptor-interacting protein (GRIP1/Tif2) and steroid receptor-coactivator protein 1 (SRC1), both being able of interacting with the AF-2 domain of the steroid receptors via their NR-boxes that are located in the middle region of the p160 proteins. After binding, they can relay their activating signal through their activation domains by recruiting secondary coactivator proteins. p/CAF can modulate gene regulation by modifying chromatin structure via its histone-acetyltransferase activity (HAT). It can interact with the glucocorticoid receptor by binding to the AF-1 domain, or via recruitment by other glucocorticoid receptor-interacting coactivators into the coactivator complex. CBP ((cyclicAMP-response element-binding protein)-binding protein) can interact directly with the AF-1 domain of the nuclear receptor or indirectly via the AF-2 domain through interactions with other gene regulators. Because of its numerous interactions with other regulatory proteins, it can integrate multiple signal inputs. The function of coactivators is thus twofold: they can integrate multiple signalling pathways, and in addition, function as bridging proteins between GR and the basal transcription machinery.

V. GLUCOCORTICOID RESPONSE ELEMENTS
The activated glucocorticoid receptor can interact with the regulatory regions of responsive genes to alter the level of gene expression. Three types of binding sites can be discriminated: glucocorticoid-response elements (GREs) and glucocorticoid-response element half-sites (GRE½s) that are involved in activation of gene expression, and negative glucocorticoid-response elements (nGREs) that repress gene expression.

1. GREs
Activation of gene transcription by the glucocorticoid receptor, involving direct binding to the DNA, is established through so called glucocorticoid-responsive elements. Based on a number of GREs, a consensus GRE has been defined as GGTACAnnnTGTTC in which the 3' half is most conserved. A GR-monomer first binds to the 3' half-site, after which the 5' half-site is occupied by a second monomer to form a DNA-bound dimer. The glucocorticoid receptor can, nevertheless, also bind the DNA as a monomer at so-called GRE-half sites (GRE½). However, a literature search on glucocorticoid-regulated genes did not reveal GRE½s that act as simple elements in activating transcription (Figure 2).
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<table>
<thead>
<tr>
<th>Fold induction</th>
<th>Sequence</th>
<th>Position</th>
<th>Accessory elements</th>
<th>Gene</th>
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</thead>
<tbody>
<tr>
<td><strong>Simple GRE</strong></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>12</td>
<td>TGTCTCT</td>
<td>-1159</td>
<td></td>
<td>Serine/threonine protein kinase (agk1)</td>
</tr>
<tr>
<td>2</td>
<td>GGACAggttTGTCT</td>
<td>-2421</td>
<td></td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>2</td>
<td>GGCTGAggttTGTCT</td>
<td>-365</td>
<td></td>
<td>82-adrenergic receptor</td>
</tr>
<tr>
<td>4</td>
<td>ACATGAggttTGTCT</td>
<td>-583</td>
<td></td>
<td>Chromogranin A</td>
</tr>
<tr>
<td><strong>Composite GRE</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>TGTAaacaggttTGTCT</td>
<td>-2495</td>
<td>2: Myc/Mac, Homsodomain</td>
<td>81-adrenergic receptor</td>
</tr>
<tr>
<td>13</td>
<td>CACACAaaaTGTGCA</td>
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<td>y-fibrinogen unit gene</td>
</tr>
<tr>
<td>13</td>
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<td>-353</td>
<td>1: Unknown (G1:ttttgagaga)</td>
<td>Interleukin-2 receptor</td>
</tr>
<tr>
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<td>-6.3</td>
<td>2 GREs</td>
<td>alpha</td>
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<tr>
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<td>-901</td>
<td></td>
<td>hCYP3A</td>
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<tr>
<td>4</td>
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<td>-3500</td>
<td>5: 1x C/EBP, 2x NF-1, 1x HNF1, 1x HNF1 or C/EBP</td>
<td>Phenylalanine hydroxylase</td>
</tr>
<tr>
<td></td>
<td>TGTCTCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>CACACAaaaTGTGCA</td>
<td>-374</td>
<td>4: 1x HNF4/COPP-TP, 1x C/EBP</td>
<td>Tyrosine aminotransferase</td>
</tr>
<tr>
<td>16</td>
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<td>-6.3</td>
<td>1x FoxA, 1x COPP-TP</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>16</td>
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<td>-6.3</td>
<td>1x unknown (P3)</td>
<td>Carbamoylphosphate synthetase</td>
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<td>6-phosphofructo-2-kinase</td>
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<td>TGTCTCT</td>
<td>5' UTR</td>
<td></td>
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<tr>
<td></td>
<td>AGAAGAaaaTGTGCT</td>
<td>-773</td>
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**Figure 2. Inventory of glucocorticoid-regulated genes acting via GREs.** Listed are the positions and sequences of GREs or GRE%28s in glucocorticoid-regulated genes, as well as their accessory elements. Fold-induction is given as the ratio of the glucocorticoid-induced reporter-gene activity over the basal activity in transient-transfection assays.

Instead, GRE%28s seem to require additional elements to mediate a glucocorticoid response. In the phenylalanine-hydroxylase gene, for instance, a number of liver-enriched transcription factors form these accessory elements 57,58. GRE%28s can, however, also act without accessory elements by making use of multiple GRE%28s. This is exemplified by the hCYP3A gene, in which two GRE%28s enable glucocorticoid-dependent gene regulation 59. In the thyrotropin-releasing hormone gene, a combination of a GRE and a GRE%28 are responsible for the glucocorticoid response 60. Thus, although simple GRE%28s have not been found in glucocorticoid-regulated genes, combinations with accessory factors, GREs, or additional GRE%28s, can render a gene glucocorticoid-inducible. It is probable that, because of the low affinity, monomeric-GR has difficulties binding the GRE half-site. When combined with other REs, cooperative interactions may facilitate binding of monomeric GR. In contrast to GRE%28s, certain glucocorticoid-inducible genes were found to contain a simple-acting GRE. When we compared these GREs with the consensus GRE, we found a matrix-
similarity score (a measure for the similarity between the query and the consensus matrix on a scale from 0.5 to 1.0) ranging from 0.79 to 0.86. The fold-induction in response to glucocorticoids is, however, only 2- to 4-fold. Thus, although these GREs exhibit a good match to the consensus GRE, glucocorticoid induction is only moderate. In a number of genes, GREs are both spatially and functionally clustered with other REs into GRUs. In contrast to simple GREs, induction in response to glucocorticoids can be increased up to 57-fold for the very active GRU of the CPS gene. However, lower inductions are also possible. Mediocre inductions of 8- to 16-fold are obtained with the GRUs for phosphoenolpyruvate carboxykinase, 6-phosphofructo-2-kinase, and phenylethanolamine N-methyltransferase, whereas the tyrosine aminotransferase and alpha-1-acid glycoprotein genes are only 3-fold upregulated by glucocorticoids. It thus seems that organisation of GREs into GRUs allows, but does not guarantee higher induction levels than seen with simple GREs. Please note that the fold inductions given in Figure 2 are obtained from in vitro transfections in which background is generally low, and thus result in higher fold inductions relative to in vivo experiments.

Many of the GRUs depicted in figure 2 are present in genes expressed in the liver. Likely, this tissue-specificity is the result of the composition of its regulatory regions. By combining GREs with tissue-specific accessory factors, the glucocorticoid-response can be limited to one or a few tissues. The CPS GRU, for instance, ensures the selective expression in hepatocytes. Amongst its composing accessory elements are the liver-enriched transcription factors C/EBP and FoxA. Transient-transfection assays with CPS GRU reporter constructs indeed only exhibit reporter-gene expression in hepatoma cells and not in e.g. fibroblasts. Thus, organisation of regulatory elements into regulatory units enables a gene to obtain a higher level of induction, and additionally, allows the integration of multiple signal inputs into one response.

2. nGREs
Glucocorticoid-response elements that negatively influence gene expression of responsive genes are referred to as negative GREs (nGREs). In this mode of regulation, direct binding of the glucocorticoid receptor to the nGRE is required. A nGRE, therefore, has a similar recognition sequence as a GRE, although the consensus sequence of a nGRE is more variable (ATYACnnTnTGATCn) than that of a GRE.
Several regulatory mechanisms for these nGREs have been reported. In the competition model, GR-binding to the nGRE interferes with other transcription factors for binding to their response elements, thereby eliciting a negative response. The different effects of glucocorticoids on the AFP gene of human and rodents exemplify this. The human AFP gene is positively regulated by glucocorticoids through a GRE located at -175 to -161, whereas rodent AFP is inhibited by glucocorticoids. Specific deviations from the human sequence make this region less similar to a consensus GRE but better matching with an HNF4 consensus sequence. In rodents, HNF4 does, indeed, bind to this sequence, but addition of dexamethasone allows competition between GR and HNF4 for this response element, and thereby inhibits expression of the rodent AFP gene \(^{69}\).

A second, more complex mode involves the interaction with other transcription factors. Here, GR binds its response element upon addition of hormone, but can only exert its repressive effect by interacting with other transcription factors or co-activators. Depending on the identity of these interacting factors, GR-binding could induce a repressive chromatin environment or elicit a different response. The former is suggested as a mechanism for the negative regulation of the corticotropin-releasing hormone gene by glucocorticoids \(^{70}\). Adjacent AP1 and GR binding sites (\(-278\) to \(-49\)) in the corticotropin-releasing hormone promoter facilitate the binding of these proteins. Although not yet tested, a possible mechanism for glucocorticoid-mediated repression might involve the recruitment of a different chromatin-modification complex depending on whether GR or AP-1 is bound, thereby modulating the level of transcription.

**VI. HORMONE RESPONSE UNITS**

1. **Similarities and differences in GRU composition**

A number of glucocorticoid-responsive genes, in which the response-elements are organised into regulatory units, have been identified. Figure 3 shows these units for genes involved in gluconeogenesis, classified according to the pathway in which they function. Looking at the composition of the different response units, there does not seem to be a general rule on how a regulatory unit is organised. They not only diverge in the number of response elements in each unit, ranging from 4 to 10, but also in the order of response elements. From the work on PEPCK \(^{71}\) and CPS \(^{61}\), it has become clear that the specific organisation of REs is of great importance for the functionality of the unit. In PEPCK, swapping of the FoxA (AF2) and COUP-TF (AF3) elements strongly reduced reporter-gene activity in response to glucocorticoids, while
in CPS no fully functional combination of response elements could be constructed other than the wild-type configuration. Besides the presence of GREs, regulatory regions of these gluconeogenic genes all contain binding sites for the liver-enriched transcription factors C/EBP and FoxA. Likely, these transcription factors fulfil specific regulatory tasks within this class of genes. Besides binding sites for these three transcription factors, additional REs are present. The CPS GRU, for instance, has an additional response element for an unknown transcription factor denoted P3. Based on the number of response elements and the number of repetitive response elements, the CPS GRU is by far the simplest unit of the units presented here (Figure 3). However, we have shown that a functional GRU can be generated that is even simpler.

**Amino acid degradation**

**Gluconeogenesis**

**Urea cycle**

**Figure 3. Schematic representation of GRUs of genes involved in amino-acid degradation, gluconeogenesis, and ammonia detoxification.** AKD= α-ketoacid dehydrogenase E2 subunit (only putative sites); PAH= phenylalanine hydroxylase; TAT= tyrosine aminotransferase; PEPCK= phosphoenolpyruvate carboxykinase; PFK-2= 6-phosphofructo-2-kinase; CPS= carbamoylphosphate synthetase.
When the P3 element, located between the FoxA and GR REs of the CPS GRU, is removed, a functional GRU can be generated when the distance between the FoxA and GR REs is 2 or 12 basepairs. Furthermore, substituting the C/EBP, FoxA, and GR REs by optimal binding sequences for these transcription factors can also circumvent the requirement for a functional P3 element to bridge the 25-basepairs distance between the FoxA and GR REs. Apparently, the CPS GRU is organised as such to allow the gene to be regulated by all four transcription factors. We therefore hypothesise that the sequence and the architectural organisation of GRU REs are determined by the required signal inputs. The role of transcription factors recurrently acting in GRUs is outlined in the section below.

2. Accessory elements in GRUs involved in gluconeogenesis

FoxA

The transcription factor forkhead box A (FoxA/HNF-3) binds as a monomeric protein to its recognition sequence and belongs to the large family of forkhead transcription factors that contain a winged-helix motif/forkhead domain. FoxA is a major constituent of GRUs in gluconeogenic genes, indicating that the functional interaction between FoxA and GR is a common mechanism for glucocorticoid-regulated gene expression. Three FoxA family members have been identified, FoxA1, FoxA2, and FoxA3 (formerly denoted HNF-3α, HNF-3β, and HNF-3γ, respectively). The three isoforms respond differently to the same stimuli. In liver, addition of dexamethasone results in a 2.5-fold increased mRNA level for FoxA1 and a 1.5-fold increase for FoxA2, whereas FoxA3 is not upregulated. Streptozotocin-induced diabetes elevated FoxA2 and FoxA3 mRNA by 1.5-fold and 1.8-fold respectively, but did not affect FoxA1 expression. In H4IIIE hepatoma cells, the C-terminal trans-activation domain of FoxA2 is involved in the glucocorticoid-dependent transcription of gluconeogenic genes.

The mechanisms by which the FoxA family members regulate transcription have only been solved partially. One mechanism involves transactivation via its transactivation domains. Besides its DNA-binding domain and nuclear localisation signal, FoxA contains four transactivation domains. However, no co-activators interacting with these domains have been identified thus far. FoxA has been shown to be phosphorylated in vivo by casein kinase I at four phosphorylation sites, but mutation of these sites had no effect on the transactivation potential of FoxA2 in co-transfection assays.
In addition to transcriptional regulation of target genes via its transactivation domains, the FoxA proteins can also activate gene expression by altering chromatin structure. The linker histones H1 and H5 that compact the nucleosome-bound DNA, have striking similarities to the FoxA proteins. The function of the linker histones is to restrict the DNA on the nucleosome surface. In contrast to histones H1 and H5, FoxA cannot compact the DNA, and thus FoxA proteins that bind to DNA on the nucleosome core and displace the linker histone, induce de-compaction of the chromatin followed by transcriptional activation. Crystallographic analysis of FoxA3 revealed that the protein bends the DNA in a 13° angle. FoxA recognises both DNA sequence and nucleosome structure. It is suggested that the ability to bind nucleosomal sites which deviate from the consensus FoxA site, is caused by the fact that the DNA is already bent on the nucleosome. Recognition of this structural site and the lack of condensing capacity generate an open configuration that allows binding of other transcriptional regulators.

**CCAAT/enhancer-binding protein**

In all known GRUs of genes involved in glucose homeostasis, C/EBP acts as an accessory factor. Also, when devoid of a GRU, C/EBP often plays an important role in the regulation of catabolic genes. For instance, the glucocorticoid-response of the arginase gene, lacking a GRE, is mediated through C/EBPβ. C/EBP proteins thus play an important role in the regulation of expression of catabolic enzymes. The C/EBP family comprises eight isoforms and are expressed in liver and adipose tissue. Mice deficient in C/EBPα suffer from hyperammonemia and hypoglycemia due to the decreased expression of ornithine-cycle and gluconeogenic enzymes. Also, C/EBPβ-knockout mice have severely impaired gluconeogenesis.

There are three mechanisms by which C/EBP proteins can interact with the glucocorticoid-signalling pathway. First, glucocorticoids up-regulate hepatic expression of both C/EBPα and C/EBPβ. Thus far, no GREs have been identified in the gene for C/EBPβ, indicating that upregulation by glucocorticoids involves a secondary mechanism. A second mechanism involves interaction between the activated glucocorticoid receptor and C/EBP either directly via its bZIP domain (Figure 4a) or via a co-activator such as CREB-binding protein (CBP) (Figure 4b and 4c). Finally, C/EBPs can recruit a chromatin-remodelling complex to the DNA, thereby inducing local chromatin modifications enabling access of other transcription factors to the DNA (Figure 4d).
Hepatocyte nuclear factor 4 (HNF4)

HNF4 is a nuclear hormone-receptor family member that acts as a positive transcriptional regulator of liver-specific genes. Although it has been suggested to be liganded by fatty acyl-CoA thioesters (fatty acids modified by acyl coenzyme A synthetase)\textsuperscript{89,89,90}, it is still referred to as an orphan receptor (a receptor for which the activating ligand is either unknown or unnecessary)\textsuperscript{41}. Like GR, HNF4 also contains an N-terminal AF-1 (A/B) domain that mediates transcriptional activation constitutively. The ligand-binding domain harbours the ligand-dependent activation function 2 (AF-2), as well as a dimerisation function. In the adult, HNF4 is expressed in liver, gastrointestinal tract, pancreas, and kidney\textsuperscript{91}. HNF4 binds as a homodimer to REs in the regulatory regions of genes expressed exclusively in liver. In addition, HNF4 can recruit the Smad3 and Smad4 proteins, suggesting transcriptional cross talk between the TGFβ- and HNF4-signalling pathways\textsuperscript{92}. 

Figure 4. Mechanisms for interaction between GR and C/EBP. a) GR and C/EBP can interact directly via the bZIP domain of C/EBP, via common co-activators (b), or via recruitment of GR into coactivator complexes (c). Alternatively, GR or C/EBP can recruit co-activators with histone acetyltransferase activity that can alter local chromatin structure and facilitate binding of other transcription factors (d).
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**COUP-TF**

COUP-TF belongs to the family of orphan nuclear receptors and can form homodimers. COUP-TF1 knockouts showed that it is an important regulator of neuronal development and differentiation, whereas COUP-TF2 plays a crucial role in the regulation of mesenchymal-endothelial interactions. Based on sequence homology with the nuclear receptors, COUP-TF contains a DNA-binding domain, a P-Box, and a ligand-binding domain. COUP-TF can act as a repressor and an activator of gene transcription. It has, for example, been shown to antagonise HNF4-dependent activation of apolipoprotein CIII expression in liver, whereas it is a transcriptional activator of PEPCK expression. Although several mechanisms have been proposed, it is still unclear how COUP-TFs alter gene expression.

**Hepatocyte nuclear factor 6 (HNF-6)**

HNF-6 belongs to the One-Cut family of transcription factors. Apart from its N-terminal transactivation domain, it also contains a Cut-Homeodomain that can interact with the CREB-binding protein (CBP). There are two mechanisms by which HNF-6 can interact with regulatory regions. First, HNF-6 can directly bind to the response elements of target genes. HNF-6 can, for instance, bind the promoter region of the FoxA2 and phosphofructo-2-kinase gene. Alternatively, HNF-6 may act as a co-activator of FoxA2. One mechanism by which HNF-6 modulates gene expression involves the recruitment of CBP/p300, since CBP/p300 can interact with HNF-6 and since co-expression of both factors increased the expression of an HNF-6-responsive reporter construct. HNF-6 and FoxA2 may specifically interact in such a way that HNF-6 acts as a co-activator of FoxA2 to enhance transcription via a FoxA2-response element, whereas FoxA2 represses the activity of HNF-6-mediated transactivation. The activation of FoxA2 transactivation is probably the result of the recruitment of CBP/p300 by HNF-6, whereas the repression imposed by FoxA2 on HNF-6-transactivation is probably caused by inhibition of the binding of HNF-6 to its response element due to interaction with FoxA2.

**Ets**

The Ets family members are expressed in several tissues amongst which are the lung, liver, kidney, and the lymphoid and haematopoietic tissues, and play a role in development and apoptosis. In addition, Ets can act as a proto-oncogene, perhaps via the regulation of a number of proteases involved in degradation of the extracellular matrix, which alter the invasive behaviour of tumors. The Ets family
of transcription factors share a so-called Ets-domain (E26 transformation specific sequence) that forms a winged helix-turn-helix structure. In addition, a subset of this family has a Pointed (PNT) domain at the N-terminal regions that forms a helix-loop-helix (HLH) motif for interaction with other proteins.

Ets1 is localised predominantly in the nucleus due to the presence of a nuclear localisation signal on the Ets domain. Many Ets-family members are targets of signal-transduction cascades, thereby changing the stability of the protein, its transactivating capacity, its interaction with other proteins, and its subcellular localisation. Ets proteins regulate gene expression by interacting with other transcription factors and co-activators on composite DNA-binding sites. Many proteins, amongst which are CBP, AP-1, Stat5, and NF-xB, have been shown to interact with Ets1 to mediate tissue-specific expression of certain genes.

3. Enhancer-promoter interactions

It is noteworthy that, apart from the GRU in the gene for the α-ketoacid dehydrogenase E2 subunit, GRUs are located several hundreds or thousands of basepairs upstream of the transcription-start site. The question, therefore, emerges how the glucocorticoid-response signal, originating from a far-upstream sequence, can be transmitted to the transcription machinery. We believe the answer lies within the presence of so-called coupling elements.

Coupling elements act by linking distant enhancers to the promoter (Figure 5) and have been described for the α-fetoprotein gene and the prostate-specific antigen gene. In the prostate-specific antigen gene, androgen-response elements, located both proximal to the promoter and in the far-upstream enhancer, act as coupling elements. Binding of the androgen receptor to both sites can link the far-upstream enhancer to the promoter by interacting with proteins in the same co-activator complex, thus forming a “bridging” complex. The linkage of the distant enhancer and the promoter-proximal coupling element allows interaction of enhancer elements with the basal transcription machinery to regulate gene transcription.

In the CPS gene we recently discovered a promoter-proximal GRE that abrogates the GRU-mediated glucocorticoid response when mutated. A mechanism similar to the prostate-specific antigen gene therefore appears to apply to the CPS gene as well, with the GR instead of the AR forming the bridging complex. The distally located GRU, thus, becomes localised in the proximity of the promoter and can facilitate the formation of a transcription-activation complex.
In the PEPCK gene, the glucocorticoid response is mediated by a GRU located 455 basepairs upstream of the transcription-start site. Earlier reports defined the PEPCK GRU as a cluster of REs at -455 basepairs (Figure 3)\(^{111}\). However, in later studies a C/EBP site located at -90 bp was also found to be crucial for the glucocorticoid response\(^ {112}\). Since this promoter-proximal C/EBP element is functionally but not spatially clustered with the GRU, this C/EBP may serve as a coupling element as well. It has been shown that the FoxA, HNF4, and COUP-TF transcription factors of the PEPCK GRU interact with the SRC-1 coactivator\(^ {113}\). Since the GRE remains mandatory even after SRC-1 has been tethered to the GRU by Gal4-fusion experiments, it was suggested that GR functions to activate the SRC-1 coactivator or facilitate the recruitment of components of the basal transcription machinery by interacting with SRC-1. However, since CBP and C/EBP do functionally interact\(^ {114}\), it is also possible that the proximally located C/EBP element of the PEPCK gene facilitates the coupling of the GRU with the proximity promoter.

Based on these findings, we submit that the use of coupling elements is a general mechanism to relay the activating signal from a distal enhancer to the basal transcription machinery.
VII. PERSPECTIVES
GRU-mediated glucocorticoid action involves the orchestrated cooperation of different transcription factors that are endpoints of regulatory pathways. GRUs, thus, act by integrating multiple signal inputs into one response. Proximal promoter-coupling elements may serve as intermediaries between distant located enhancers and the transcription machinery, thereby providing a mechanism to convert the activating signal into transcriptional activation. Our results and data from other model systems have indicated that it might be a general mechanism, requiring different proteins in each system.

Several questions remain: What other transcription factor-binding sites, associated with gluconeogenesis can function as coupling elements to establish a bridging complex? Which co-activator proteins act in such complexes? Since CBP has been reported to function as a molecular scaffold, it is a likely candidate. Does differential regulation occur only at the level of transcription-factor binding to the GRU or is additional regulation in the formation of the bridging complex possible? The answers to these and other questions concerning nuclear regulatory factors should help elucidate the mechanisms of glucocorticoid signalling.
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