Chapter 3
Mechanisms of Glucocorticoid-Regulated Gene Transcription

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Abstract One fascinating aspect of glucocorticoid signaling is their broad range of physiological and pharmacological effects. These effects are at least in part a consequence of transcriptional regulation by the glucocorticoid receptor (GR). Activation of GR by glucocorticoids results in tissue-specific changes in gene expression levels with some genes being activated whereas others are repressed. This raises two questions: First, how does GR regulate different subsets of target genes in different tissues? And second, how can GR both activate and repress the expression of genes?

To answer these questions, this chapter will describe the function of the various "components" and how they cooperate to mediate the transcriptional responses to glucocorticoids. The first "component" is GR itself. The second "component" is the chromatin and its role in specifying where in the genome GR binds. Binding to the genome however is just the first step in regulating the expression of genes and transcriptional regulation by GR depends on the recruitment of coregulator proteins that either directly or indirectly influence the recruitment and or activity of RNA polymerase II. Ultimately, the integration of inputs including GR isoform, DNA sequence, chromatin and cooperation with coregulators determines which genes are regulated and the direction of their regulation.

Keywords Transcription • Coregulators • Chromatin • Cis-regulatory elements • Glucocorticoid receptor

Structure of the Glucocorticoid Receptor

Although glucocorticoids have been used clinically from the 1940s [1], it wasn't until 1984 when the coding sequence for its receptor was initially isolated from rat [2] and soon after its human homolog was cloned [3]. The human gene coding for GR

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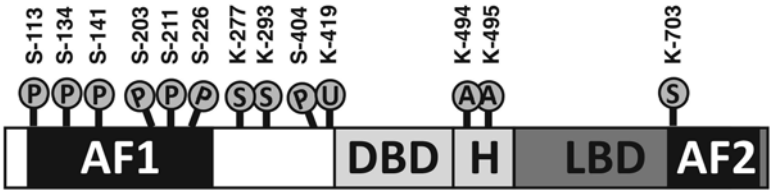

Fig. 3.1 Domain structure of GR and sites of post-translational modifications. Shown are the functional domains of GR: The activation function 1 and -2 (AF1, AF2), the DNA-binding domain (DBD), hinge region (H) and ligand-binding domain (LBD). Also shown are post-translational modifications (phosphorylation (P); acetylation (A); ubiquitination (U); sumoylation (S)) of either Serine (S) or Lysine (K) residues. Amino acid numbering refers to human GR

consists of nine exons and can produce a variety of different gene products through alternative splicing, alternative translational initiation and by post-translational modifications. Here we describe the functional domains of GR and how alternative splicing, translational initiation and post-translational modifications generate receptor isoforms with distinct expression profiles and target genes.

Functional Domains of the Glucocorticoid Receptor

A combination of biochemical (limited proteolysis, [4]) and molecular biological (mutagenesis and domain fusions) approaches have uncovered that the glucocorticoid receptor is a modular protein with several functional domains (Fig. 3.1).

The N-terminal domain of the glucocorticoid receptor contains the activation function 1 domain (AF1, amino acids 77–262, throughout amino acid numbering refers to human GR), which is involved in transcriptional regulation [5]. In contrast to the AF2 domain (see below), the AF1 domain is constitutively active meaning that its activity does not rely on the presence of hormone [5]. GR-dependent transcriptional regulation critically depends on its interaction with several coregulator proteins that either directly or indirectly recruit or influence the activity of RNA polymerase II (the role of coregulators in transcription is described in section "Transcriptional Regulation by GR"). For the AF1 domain these interaction partners include p160, TIF2, DRIP/TRAP and TBP [6]. How these proteins interact with the AF1 domain is largely unknown. No clear interaction domains have been identified in either GR or in the proteins interacting with AF1 and computational predictions and experimental approaches indicate that large parts of the AF1 domain are intrinsically disordered [7]. This may allow the AF1 to adopt different conformations to create interaction surfaces for a variety of coregulators.

DNA binding by GR is mediated by the DNA binding domain (DBD, amino acids 420–480), which is conserved across steroid hormone receptor proteins. GR can bind as a homodimer to DNA sequences consisting of inverted repeats of a loosely defined recognition sequence separated by a three base pair spacer (Fig. 3.2) [8]. The three-dimensional structure shows that the DBD contains several alpha-helices.

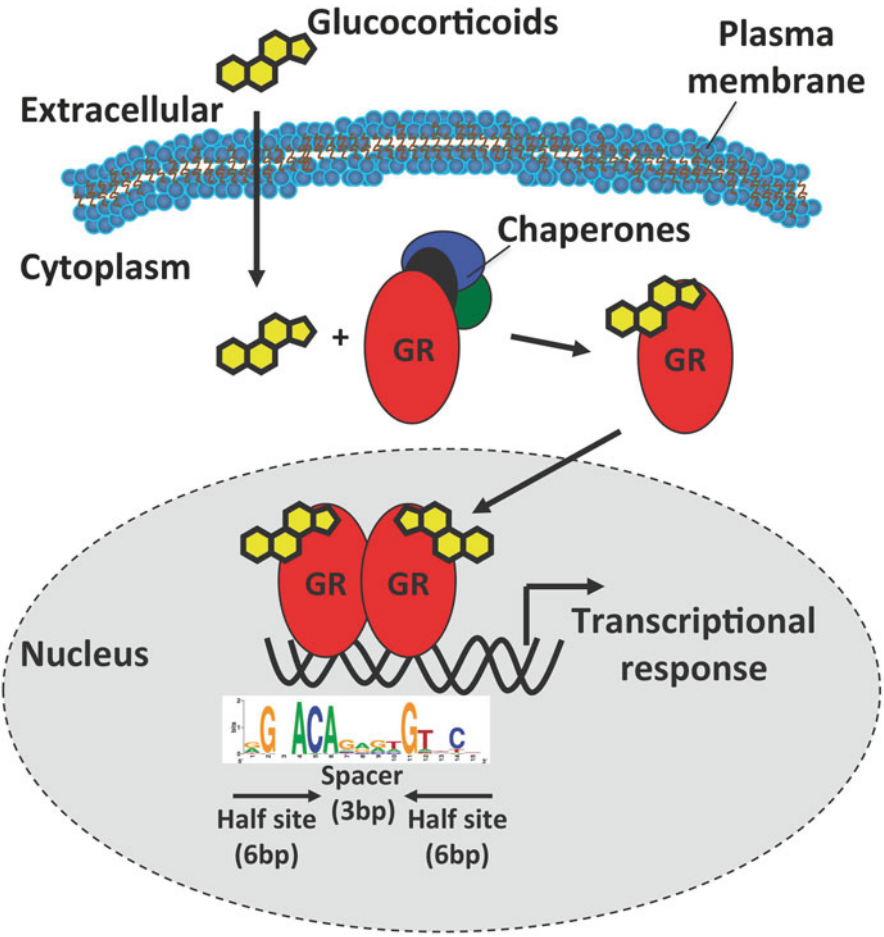

Fig. 3.2 Classical GR signaling pathway. Upon ligand binding, cytoplasmic GR dissociates from chaperone proteins and translocates to the nucleus where it interacts with specific DNA sequences to control the expression of associated target genes

One helix mediates base-specific DNA contacts whereas an alpha-helix at the C-terminus of the DBD makes several non-specific phosphate backbone and minor groove contacts [9, 10]. Two zinc-fingers ascertain proper folding of the DBD to coordinate DNA recognition and dimerization [9, 11, 12]. Like other domains, the DBD interacts with several coregulators including JDP1, JDP2, HMG1, HMG2, GT198 and SET/TAFI-β(beta) [13–16]. In addition to direct DNA binding, GR can also be tethered to the DNA for example via its interaction with activator protein 1 (AP1), NFκ(kappa)B or STAT3 (Fig. 3.3) [17–22]. Interestingly, also here the DBD appears responsible for tethered DNA interactions by directly interacting with the c-Jun/c-Fos or p65 subunits of AP1 and NFκ(kappa)B respectively [17, 21, 23].

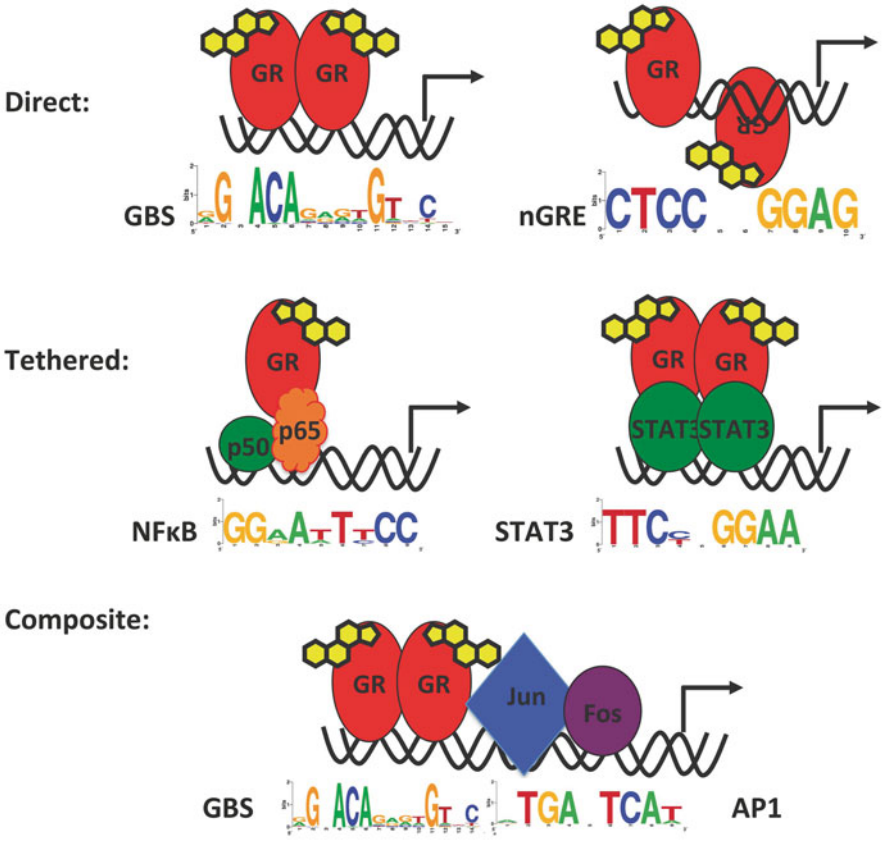

Fig. 3.3 DNA binding by GR. Glucocorticoid-activated GR can interact with DNA either directly (*top*), indirectly via tethering (*middle*) or can bind to composite elements where it engages in cross-talk with neighboring DNA-bound transcriptional regulatory factors (*bottom*)

Arguing for an important role of the DBD in tethered DNA binding, mutations in this domain interfere with GR's function as a transcriptional repressor at sites where it is tethered to the DNA by either AP1 or NFκ(kappa)B [17, 23].

C-terminal to the DBD the hinge region connects the DBD to the ligand binding domain (LBD). The LBD consists of 12 alpha helixes [24] and ligand binding is facilitated by several alpha helixes that together form a hydrophobic pocket [24]. Furthermore, the LBD harbors a second dimerization domain, sequences involved in nuclear translocation upon hormone binding and the activation function 2 (AF2 domain), which mediates the interaction with several coregulators (reviewed in [25]). In the absence of ligand, GR is predominantly cytoplasmic where the interaction of the LBD with chaperone proteins such as hsp90 and p23 keep GR in a hormone-binding competent state (Fig. 3.2) (reviewed in [26]). Ligand binding results in Hsp90 dissociation, nuclear translocation and conformational changes in helix 12

(Fig. 3.2) [26]. These conformational changes facilitate the interaction of the AF2 domain with a variety of coregulators containing LXXLL motifs [24, 26] including p160 coactivator family members SRC1 and GRIP1 [27, 28].

Although the domains of GR can function in isolation, recent studies indicate that the domains of nuclear hormone receptors are both functionally and structurally connected [29–32]. These domain-connections can be rewired depending on the context in which GR is active and accordingly, different combinations of GR domains are required to regulate the expression of individual genes [33].

Creating Functional Diversity: Glucocorticoid Receptor Isoforms and Post-translational Modifications

Although a single gene (*NR3C1*) codes for the glucocorticoid receptor protein, this gene can give rise to several isoforms with unique expression profiles [34, 35]. In addition, post-translational modifications of these isoforms further expand the diversity of responses to glucocorticoids. Besides the predominant GR isoform GRα(alpha), alternative splicing of GR can generate at least four additional isoforms: GRβ(beta), GRγ(gamma), GR-A and GR-P [25]. GRβ(beta) differs from GRα(alpha) in its LBD and is unable to bind hormone [36]. The GRβ(beta) isoform appears to be transcriptionally inactive and can antagonize the activity of GRα(alpha) [37]. Accordingly, increased GRβ(beta) levels have been linked to glucocorticoid resistance in a variety of diseases including asthma, rheumatoid arthritis and acute lymphoblastic leukemia [37]. Use of an alternative splice-donor site generates the GRγ(gamma) isoform, which differs from GRα(alpha) in having a single additional Arginine inserted in the DBD [38]. The Arginine insertion results in gene-specific effects with most genes being unaffected, whereas some genes are regulated more strongly and others more weakly [30]. Consistent with a reduced activity towards certain target genes, GRγ(gamma) has been linked to glucocorticoid resistance in childhood acute lymphoblastic leukemia and small cell lung carcinoma cells [39, 40]. The GR-A and GR-P isoforms lack exons encoding the LBD and consequently lack the ability to bind ligand [41]. Similar to GRβ(beta) and GRγ(gamma), GR-P can antagonize the transcriptional activity of GRα(alpha) and has been linked to glucocorticoid-resistance [42].

Additional GR isoforms are produced as a consequence of alternative translational initiation, which generates GR proteins with shorter N-terminal domains [43]. These translational isoforms differ in their tissue-specific expression and the transcriptional programs they initiate [44]. Another mechanism that can generate functional diversity are post-translational modifications. Such modifications can alter the function of GR and include phosphorylation, acetylation, sumoylation and ubiquitination (Fig. 3.1) [25]. One example of a post-translational modification that influences GR activity is the phosphorylation of Serine residues in the N-terminus of GR. The phosphorylation modulates GR's interaction with coregulators and

differentially affects its activity towards individual target genes [45]. Another example is acetylation of GR by CLOCK, a histone acetyltransferase (HAT) involved in circadian rhythm. The CLOCK-dependent acetylation of multiple Lysines in the hinge region of GR interferes with DNA binding resulting in changes in the expression level of a subset of GR target genes [46, 47].

Together, alternative splicing, translational initiation and post-translational modifications generate a variety of GR variants with different target genes. Consequently, differences between cell types and tissues in the expression level of these isoforms and of the enzymes responsible for post-translational modifications likely contribute to the highly tissue-specific effects of glucocorticoids.

Chromosomal Binding of GR

Hormone binding by GR results in nuclear translocation and allows the receptor to bind to specific genomic sequences (Fig. 3.2). The binding of GR to glucocorticoid response elements (GREs) constitutes an essential first step in the regulation of the expression of associated target genes. Here we discuss the contributions of DNA sequence elements and the chromatin landscape in guiding GR to its appropriate genomic destination.

Binding to the Genome: "Classical" GR Binding Sequences

The first described "classical" mode of DNA binding is for liganded GR to associate as a dimer to GR binding sequences (GBSs) [8]. GBSs are typically imperfect palindromic hexameric half-sites separated by a 3bp spacer (Fig. 3.2). Historically, mostly for practical reasons, studies to identify regulatory sequences exploited by GR to regulate target genes were focused on promoter regions and have uncovered numerous promoter-proximal GBSs [48–50]. In support of a role of these GBSs in the regulation of associated target genes, genomic regions that harbor a GBS as well as simply the 15bp GBS are sufficient to facilitate GR-dependent transcriptional activation when localized upstream of heterologous promoters [10, 49]. However, up until recently it was unclear whether promoter-proximal binding by GR is the exception or the rule that governs genomic binding and the control of target gene expression. Technological advances that combine chromatin immunoprecipitation (ChIP) with next generation sequencing (ChIP-seq) allow the unbiased genome-wide identification of GR binding sites [51, 52]. Several ChIP-seq studies have revealed that promoter-proximal binding by GR appears to be the exception and that the majority of GR binding is at promoter-distal locations [53–56]. One representative study showed that for genes that are up regulated in response to glucocorticoid treatment (likely GR target genes), 50 % of the binding sites were located at a distance greater than 10 kb from the transcriptional start-site (TSS) [54]. Even more

striking, for down regulated genes the median distance to the TSS was >100 kb [54]. The finding that only the minority of GR binds promoter-proximal is not specific for GR but is also seen for related hormone receptors including ER, PPAR and AR [55, 57, 58]. This suggests that long-range regulation by GR and other hormone receptors might be responsible for the regulation of a large fraction of target genes. In support of this idea, a study using chromatin conformation capture showed that the promoter of the GR-regulated gene *Ciz1* was contacted by a GR binding region located nearly 30 kb away [59].

Bioinformatical analysis of genomic regions bound by GR shows that the canonical 15 bp GBS is highly enriched at such binding sites with one study reporting that 58 % of the bound regions contains a GBS [56]. This underscores the important role of the canonical GBS in guiding GR to specific genomic locations. It does however also hint at the existence of alternative sequences that facilitate GR binding at the remaining 42 % of GR-bound regions.

Binding to the Genome: Other Sequences

Several ChIP-seq studies made the striking observation that only a fraction of all GR binding regions appears to contain the canonical 15 bp GBS [54, 56]. This indicates that GR may be able to bind to very degenerate sequences with the assistance of another transcriptional regulatory factor. Moreover, sequences other than the canonical GBS might be able to recruit GR to specific genomic loci (Fig. 3.3). Such sequences could either bind proteins that tether GR to the DNA or alternatively GR might be able to interact directly with a broader spectrum of DNA sequences. In support of this, studies with the hormone-repressed gene *POMC* uncovered GR-bound sequences that resemble the canonical GBSs somewhat but lack similarity to the consensus motif at key positions [60]. Interestingly, whereas regulation from canonical GBSs is typically associated with activation of transcription, the promoter region of the *POMC* gene mediated transcriptional repression when fused to a luciferase reporter gene and was therefore called negative glucocorticoid response element (nGRE). This repression was lost when the GBS-like sequence was changed to resemble a canonical GBS [60]. In isolation however, this sequence failed to confer repression arguing that its function relies on other functional elements present at the *POMC* promoter [60]. Another class of sequences that has been proposed to directly interact with GR are inverted repeats of CTCC that have a spacing of either 0, 1 or 2 base pairs [61]. These sequences are associated with genes that are repressed by GR. Notably, binding of GR to canonical GBSs strictly requires a 3 bp spacer to position two GR molecules such that they can effectively dimerize [9]. The variable spacing for these nGREs suggests that dimerization might not be required at these nGREs and accordingly structural studies suggest monomeric GR-binding to the half sites (Fig. 3.3) [62].

Together these studies suggest that GR is able to interact directly with a variety of sequence motifs to control the expression of associated target genes.

Binding to the Genome: Tethered Binding

The absence of canonical GBSs in ChIP-seq peaks can also be explained by tethered DNA binding by GR (Fig. 3.3). Tethered GR binding has predominantly been linked to transcriptional repression and has been proposed for several transcriptional regulatory factors including NFκ(kappa)B [21], AP1 [18–20], STAT3 [22, 63] and NGFI-B [64]. For NFκ(kappa)B, the p65 (RelA) subunit physically interacts with GR [21] and recruits GR to NFκ(kappa)B response elements [65]. The ability of GR to repress from NFκ(kappa)B sites can be recapitulated using reporters plasmids simply harboring NFκ(kappa)B sites driving the expression of a luciferase reporter gene arguing that tethered binding to NFκ(kappa)B mediates the repressive effects of GR [66]. Genome-wide studies using ChIP-seq, showed that co-treatment of cells with dexamethasone, a synthetic GR ligand, and with TNFα(alpha) to activate NFκ(kappa)B resulted in GR binding to approximately a thousand additional genomic regions when compared to the binding profile when cells were treated with dexamethasone alone [67]. These additional binding regions are enriched for NFκ(kappa)B binding sites suggesting that tethered binding might occur quite frequently [67]. However, it could also be that part or all of the gained binding is a simple consequence of NFκ(kappa)B-induced changes in chromatin accessibility that makes previously inaccessible GR binding regions available.

AP1 is another factor that physically interacts with GR [18] and has been implicated in tethering GR to DNA [68]. Similar to the observation for NFκ(kappa)B, tethered binding by AP1 is linked to transcriptional repression. This repression can be recapitulated using a luciferase reporter that contains a single copy of the AP1 consensus sequence driving expression of a luciferase reporter gene [69]. Other proteins implicated in tethering GR to the DNA are members of the signal transducer and activator of transcription (STAT) family. GR physically interacts with several STAT proteins including STAT1 [70], STAT3 [63] and STAT5 [71]. Genome-wide profiling of STAT3 and GR binding suggests that GR may be tethered to the DNA by STAT3 at about 300 genomic binding sites and that such binding events are almost exclusively associated with transcriptional repression by GR [22].

Combinatorial Binding by GR and Regulation

Binding sites for GR in the genome are not present in isolation but are surrounded by sequence motifs that can be occupied by other transcriptional regulatory factors (Fig. 3.3). Accordingly, analysis of GR ChIP-seq peaks shows a cell-type specific overrepresentation of various sequence motifs [56]. Recent studies underscore the important role of combinatorial binding in transcriptional regulation by GR and for transcriptional regulatory factors in general [72, 73]. The study by Siersbaek and coworkers analyzed five transcriptional regulatory factors involved in adipogenesis including GR (out of the more than a thousand transcriptional regulatory factors encoded in the human genome) [72]. ChIP-seq of these factors showed combinatorial

binding of GR with at least one other factor for >93 % and simultaneous binding of all 5 factors for 25 % of all GR binding events [72]. These "hotspots" of transcriptional regulatory factor binding were also found by the encode consortium that looked at >100 transcriptional regulatory factors [73, 74]. The co-occurrence of a GR binding site with recognition sequences for "partner" transcriptional regulatory factors can give rise to a broad spectrum of signaling cross-talk. A commonly observed type of cross-talk is a synergetic interaction between GR and other transcription factors. For example, knockdown of C/EBPβ(beta) results in a reduction of GR binding at co-occupied sites whereas binding at control sites that are not co-occupied are not affected [72]. The knockdown of factors co-occupying "hotspots" revealed a highly cooperative nature of transcriptional regulatory factor binding at these sites [72]. Synergetic interactions likely reflect at least in part effects of chromatin (chromatin accessibility) where several transcriptional regulatory factors cooperate to keep genomic sites accessible. This might explain the many synergetic interactions with other transcriptional regulatory factors that have been described for GR which include SP1, NF1, STAT3, COUP-TFII and AP1 [75–78].

The cross-talk between GR and other transcriptional regulatory factors at combinatorial binding sites can also be antagonistic. For example, at the osteocalcin promoter, the GR binding site overlaps the TATA box and GR binding thereby antagonizes TFIID binding to the TATA box and transcriptional initiation [79]. Another example of an antagonistic interaction between GR due to overlapping binding sites is found at the prolactin gene where the GR binding sites overlaps site for Oct1 and Pbx1 [80]. GR can also antagonize the activity of other factors via non-overlapping binding sites as was shown for the glutathione S-transferase A2 gene [81]. Here binding of GR to a GBS-like sequence results in the recruitment of the transcriptional co-repressor SMRT to repress C/EBP- and NRF2-mediated activation [81]. For the mouse proliferin gene, depending on the composition of the proteins that bind to the dimeric AP1 binding site, GR can either act antagonistically or synergistically [82].

The complex nature of interactions between GR and other transcriptional regulatory proteins illustrates the complexity of signaling cross-talk occurring at composite elements. This complexity can potentiate the ability of GR to regulate genes in a cell type specific manner and to tailor its activity towards individual genes. Gene-specific effects can for instance be a consequence of differences in the local sequence of the GR binding site. Similarly, the cell-type specific expression and binding of transcriptional regulatory factors that engage in synergistic interactions with GR can explain tissue-specific effects.

DNA Binding: Influence of Chromatin Structure on GR Binding

Another fascinating fact that the genome-wide analysis uncovered is that the genomic binding pattern of GR shows little overlap (<5 % [53]) between cell-types [56], and personal unpublished results). The highly tissue-specific binding by GR

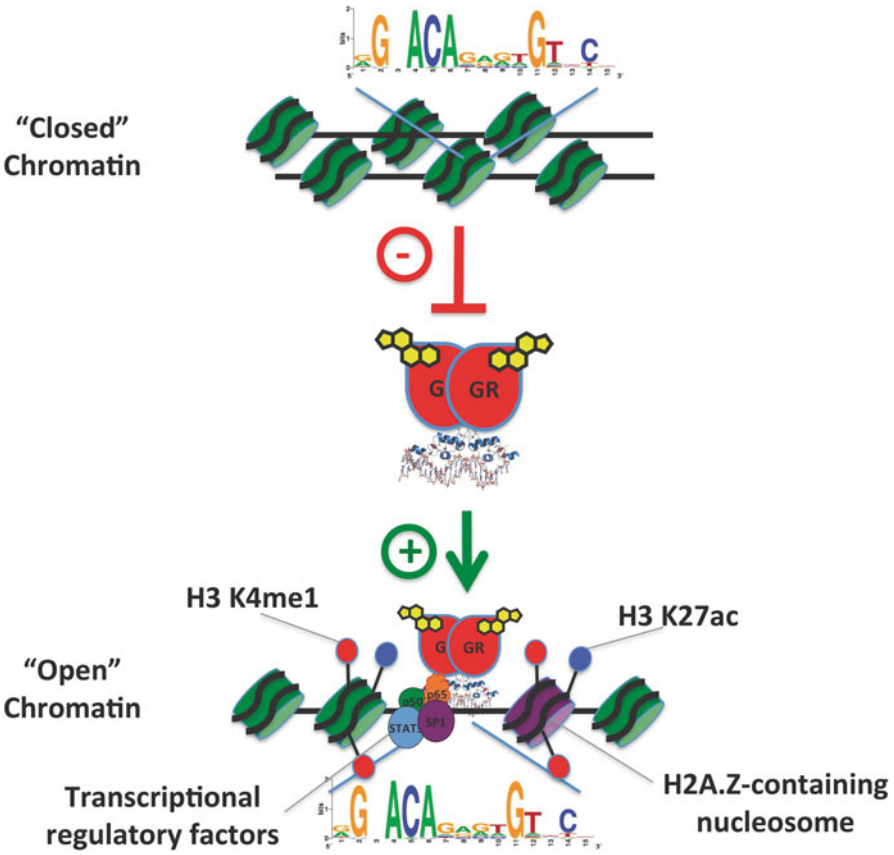

Fig. 3.4 Chromatin features influence DNA binding by GR. Chromatin features either negatively or positively correlating with genomic DNA binding by GR. Negative: closed chromatin (*top*). Positive: closed chromatin, nucleosome free DNA, enrichment for the H2A.Z histone variant, presence of other transcriptional regulatory factors and of histone modifications H3K4me1 and H3K27ac (*bottom*)

indicates that the sequence of GR binding sites alone is insufficient to explain where in the genome GR binds. Thus, DNA sequence specifies where in the genome GR could bind and other inputs including the chromatin landscape are needed to specify where GR actually does bind (Fig. 3.4). One aspect of the chromatin landscape that appears to be a major contributor is chromatin-accessibility as assayed by DNase-I accessibility assays [53, 78]. These studies showed that the majority of GR binding occurs in genomic regions that are DNase-I accessible ("open") prior to hormone treatment [53, 78]. Interestingly, which regions of the genome are actually "open" appears to be highly cell-type specific [53, 73]. So an emerging picture is that

cell-type specific "chromatin-accessibility factors", other than GR, specify which regions of the genome are "open" and thereby where in the genome GR can bind and which genes it can regulate. One such "chromatin-accessibility factor" is AP1, whose binding, according to one study, overlaps with >50 % of GR binding sites [78]. Consistent with a role in facilitating GR binding, dominant negative AP1 and depletion of AP1 levels by siRNAs resulted in reduced chromatin accessibility and a loss of GR binding at co-occupied sites [78]. Another factor linking chromatin accessibility and steroid receptor binding is the forkhead box A1 (FoxA1) protein. FoxA1 induces DNase-I hypersensitivity [83, 84] indicative of open chromatin and facilitates GR binding [83]. Conversely, depletion of FoxA1 results in a redistribution of genomic estrogen receptor (ER), androgen receptor (AR) and GR binding [85, 86]. However, for ER depletion of FoxA1 results in an almost complete loss of ER binding [85] whereas for AR and GR FoxA1 depletion results in a redistribution of binding [86] indicating that the role of FoxA1 for GR and AR is more complex than simply facilitating access to the genome.

The observation that transcriptional regulatory factors typically bind together to "hotspots" and mutually stimulate genomic binding [72] suggests that they might cooperatively keep certain regulatory regions open. This can also explain the tissue-specific binding patterns observed for GR due to cell-type-specific expression of these cooperation partners. Notably, not all GR binding occurs at open regions and for a subset of binding events GR appears to act as a "chromatin-accessibility factor" [53, 78]. Analysis of sequence motifs for closed chromatin GR binding sites showed that binding to these sites is mediated by GBSs with high motif scores [87] suggesting that high-affinity binding might be a prerequisite for GR binding at closed chromatin. Interestingly, whereas GR binding sites in "open" chromatin show little overlap between cell types, binding at "closed" sites is often shared between cell types [87] indicating that for these sites GR might not rely on other factors for binding.

Other chromatin features linked to GR binding are the presence of nucleosomes, the post-translational modification state of nucleosomal histones and the presence of histone variants. GR binding sites are enriched for several chromatin features linked to enhancers including monomethylation of histone H3 Lysine 4, acetylation of histone H3 Lysine 27 and enrichment of the histone variant H2A.Z [88, 89] and unpublished data from my group). The enrichment profile of these histone modifications shows a bimodal peak flanking the site of GR-binding, which indicates that GR typically binds to DNA located between two nucleosomes (Fig. 3.4) [88]. However, despite the significant correlation between histone modifications and GR binding, future studies are needed to determine if and how these are causatively connected.

In conclusion, the integration of DNA sequence information, cooperation with other transcriptional regulatory factors and chromatin features appears to determine where in the genome GR binds and ultimately which genes it regulates in a particular cell type.

Transcriptional Regulation by GR

The transcriptional process begins with the recruitment of RNA polymerases to the transcriptional start site (TSS) by the pre-initiation complex (PIC). After recruitment, the RNA polymerases proceed through distinct steps of the transcription cycle: initiation, elongation and termination. RNA polymerases are multi-protein complexes, which change their composition and/or carry different modifications dependent on the step in the transcription cycle. For example, RNA polymerase II is differentially phosphorylated in the C-terminal tail domain (CTD) of its largest subunit dependent on whether it is initiating, elongating or terminating (reviewed in [90]). Gene regulation depends on the action of transcriptional regulatory factors, like GR. GR can exploit a broad spectrum of mechanisms to influence the expression level of genes. Such mechanisms include influencing RNA stability [91–93], sequestering or influencing the activity state of other transcriptional regulatory factors by protein:protein interactions [94, 95] that thus does not require direct interactions of GR with DNA or with the RNA polymerase machinery. Here however, we will focus on transcriptional effects in response to glucocorticoids that involve DNA binding and RNA polymerase II. GR may affect the state of RNA Polymerase II directly (e.g., the phosphorylation state of the CTD or the assembly of the PIC). Alternatively, GR can modulate RNA polymerase II's regulatory role indirectly by recruiting coregulators such as histone modifying enzymes, chromatin remodelers or the mediator complex that bridges the interaction with RNA polymerase II (Fig. 3.5). GR can either increase the transcription rate (hence acting as an activator) or can reduce—or even eliminate—transcription (acting as a repressor). This paragraph presents an overview of different classes of coregulators and their role in mediating the transcriptional effects of GR.

Interaction with Coregulators: Interactions with the Basal Transcriptional Machinery

Perhaps the most straightforward way for GR to influence transcription is by interacting directly or indirectly with components of the basal transcriptional machinery (Fig. 3.5). A direct interaction of the GR's AF1 domain with TBP, which is part of the TFIID component of the pre-initiation complex, suggests that GR can promote transcriptional initiation by recruiting TFIID to promoters of target genes [96]. Furthermore, GR interacts with p300/CBP, which in turn interacts with TFIIB, another component of the pre-initiation complex, and thereby indirectly linking GR to the basal transcriptional machinery [97–99]. The glucocorticoid receptor can also recruit RNA polymerase II via its interaction both physically and functionally with components (MED1 and MED14) of the mediator complex, which interacts with the CTD of RNA polymerase II [100, 101].

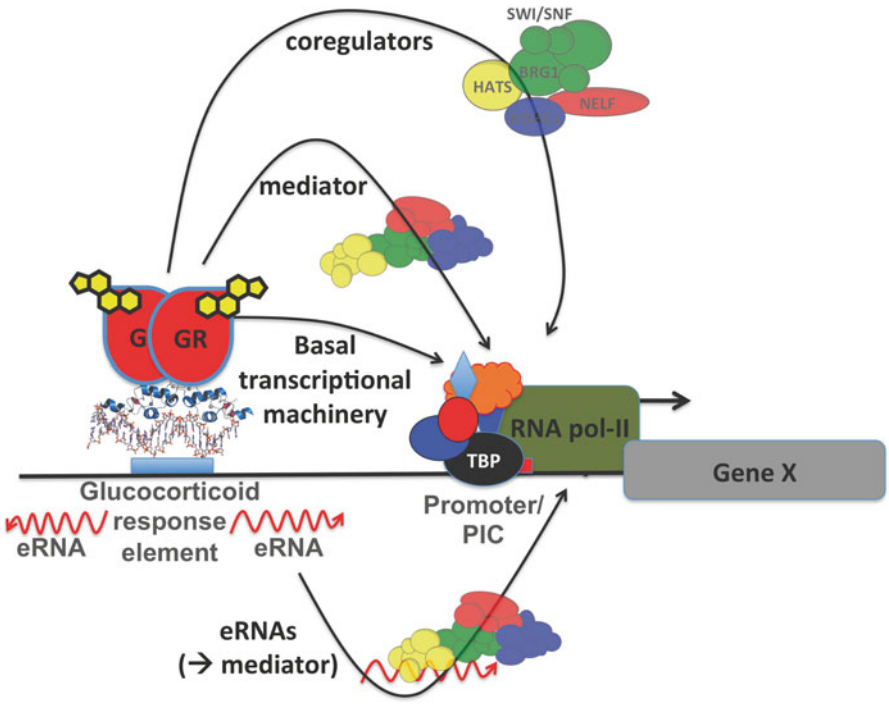

Fig. 3.5 Coregulators and their role in GR-dependent regulation of promoter activity. Overview of interacting coregulators (proteins and RNA) of GR that can either directly or indirectly influence the recruitment or activity of RNA polymerase II and thereby the transcriptional output. *Abbreviations*: *HATS* histone acetyltransferases, *HDACs* histone deacetylases, *NELF* negative elongation factor, *PIC* pre-initiation complex, *TBP* TATA-binding protein, *eRNA* enhancer RNA

Transcriptional control by GR is also exerted at the level of transcriptional elongation, the step in the transcription cycle downstream of transcriptional initiation. For example, GR can interact with proteins that stimulate elongation (elongation factor RNA polymerase (ELL)), resulting in increased levels of transcript [102]. Conversely, at the IL8 gene GR displaces p-TEFb, a factor that stimulates elongation [65]. The displacement prevents the p-TEFb-dependent Serine 2 phosphorylation of the CTD of RNA polymerase II and consequently reduces transcriptional elongation at the IL8 gene [65, 103]. Furthermore, GR interacts with suppressors of elongation (negative elongation factor (NELF), [104]). An elegant study in macrophages shows that GR can either repress the expression of genes at the level of RNA polymerase II recruitment or by recruiting NELF, which results in a pausing of RNA polymerase II [104]. In agreement with a role for NELF in mediating the effects of GR, repression was specifically lost for the elongation-controlled genes in NELF-deficient macrophages [104]. Together these studies indicate that GR directly or indirectly contacts components of the basal RNA polymerase II machinery and thereby can influence gene expression by affecting different stages of the transcription cycle.

Coregulators That Influence Chromatin Structure and Histone Modification States

In eukaryotes DNA accessibility and chromatin structure play an important role in specifying the expression level of genes. Eukaryotic genomes are packaged into chromatin, whose basic repeating unit is the nucleosome [105, 106]. Nucleosomes form by wrapping 147 base pairs of DNA around an octamer of the four core histones (H2A, H2B, H3 and H4) and can be found approximately every 200 base pairs throughout the genome [107]. Their presence affects all DNA-dependent processes, including DNA-repair, DNA replication and transcription. For instance, *in vitro* a chromatinized DNA template prevents RNA polymerase II from initiating transcription [108]. Even before the identification of the coregulators responsible, studies of promoters of hormone-activated genes showed that transcriptional regulation by GR is tightly coupled to chromatin remodeling [109, 110]. Especially studies with the mouse mammary tumor virus (MMTV) have been instrumental in dissecting the steps needed for transcriptional activation (reviewed in [110]). These steps include the recruitment of chromatin modifying enzymes, nucleosome repositioning and changes in sensitivity to nucleases, ultimately resulting in the recruitment of RNA polymerase II. GR-dependent chromatin remodeling is mediated by its interaction with a variety of coregulators that modify chromatin structure and thereby indirectly the recruitment of RNA polymerase II. The first class of GR-interacting chromatin modifiers are members of ATP-dependent chromatin remodelers that can move and remove nucleosomes. Specifically, GR interacts with the ATP-dependent chromatin remodeling complex SWI/SNF [98, 111]. This interaction is mediated by BAF proteins that are part of the SWI/SNF complex [112]. The SWI/SNF complex contains one of two possible core ATP-ase subunits [110] Brm or BRG1 and a physical and functional connection between both ATPase subunits and GR activity has been shown [89, 113, 114]. The interaction with the SWI/SNF complex is essential for GR-dependent transcriptional activation of the MMTV promoter [98]. Here, the SWI/SNF complex repositions nucleosomes to allow other transcriptional regulatory factors and TBP to bind and thereby facilitates the assembly of the pre-initiation complex at the promoter (reviewed in [110]). For endogenous genes, the effects of disrupting Brm or BRG1 activity, by either dominant negative versions of these proteins or by knocking down their expression using siRNA, results in gene-specific effects with some genes being affected whereas others are not [89, 114]. The mechanisms responsible for the facultative requirement for Brm and BRG1 are unknown, but might reflect the fact that for certain genes alternative mechanisms ensure appropriate nucleosome positioning and transcriptional initiation.

The second class of chromatin modifying enzymes that interact with GR are enzymes that post-translationally modify histones. These histone modifications can act as recognition signals for proteins [115]. For example, trimethylated Lysine 4 of histone H3 is recognized by TFIID providing a direct link between histone modifications and the basal transcriptional machinery [116]. Additionally,

histone modifications might influence transcription by loosening the chromatin. This occurs when Lysines are acetylated which removes its positive charge thereby reducing the affinity between DNA and histones [117]. One coregulator that acts as a coactivator of GR is the histone acetyltransferase p300 [118]. Conversely, enzymes that remove acetylation marks, histone deacetylases (HDACs), can act as corepressors of GR [119]. Examples of HDACs or complexes containing HDACs that interact with GR are NcoR, SMRT and HDAC2 [61, 119]. Although the activities of HATs and HDACs might be a consequence of "loosening" the chromatin, their role is likely to be more complex. One added level of complexity is that in addition to histones, these enzymes can also modify transcriptional regulatory factors, chaperones like hsp90 and coregulators [47, 120]. For example, acetylation of GR by either CLOCK or GCN5 interferes with GR's ability to interact with DNA [47, 121]. Further illustrating the complexity of the interaction, the GR-interacting coregulator GRIP1 acts as a coactivator for some GR target genes whereas it acts as a corepressor at others [122]. In addition to coregulators that modify the acetylation state of Lysines, GR also interacts with histone modifying enzymes CARM1 and G9a that methylate respectively Arginine or Lysine residues of histones and other proteins [123, 124].

In conclusion, genomic binding by GR coordinates the recruitment of a large variety of coregulator proteins. These coregulators specify the activity as well as the direction of the transcriptional responses to glucocorticoids at individual target genes. However, the underlying mechanisms responsible for the context-specific requirement of coregulators remain largely unknown. One possible explanation could be that the combinatorial binding of GR and another factor creates an interaction surface for coregulators that is not present when these factors bind in isolation. Furthermore, the DNA binding site responsible for GR recruitment appears to play an important role as tethering sites and non-canonical GBSs are typically associated with repression and preferentially recruit corepressors whereas canonical GBSs direct the assembly of regulatory complexes that usually activate transcription. The role of the DNA sequence might in part be explained by the fact that DNA induces sequence-specific conformational changes in the DBD of GR [10, 125]. These conformational changes could be propagated to domains of GR engaged in protein:protein interactions thereby explaining the context specific signaling cross-talk between GR and coregulators.

Concluding Remarks and Future Perspectives

The past decades have generated a wealth of mechanistic insight into how GR orchestrates the transcriptional response of cells and tissues to glucocorticoid hormones. It is becoming increasingly clear that these responses are highly context-specific and that chromatin plays a key role in dictating which transcriptional program is initiation in a particular cell type. In addition to the cell-type-specific

effects, GR also appears to have highly gene-specific effects within a cell. This might complicate research as there are perhaps few universally applicable operating principles for GR in transcriptional regulation. It also provides an opportunity to try to activate GR in a targeted way that may selectively affect the expression of a subset of genes and thereby might result in therapeutic usage of glucocorticoids with fewer side effects. One approach in this regard has been to develop synthetic GR ligands with selective activities [126, 127]. Several such ligands do indeed regulate subsets of GR target genes [126, 127]. However, if and to what extend ligands can be identified that display such selectivity towards the therapeutically relevant target genes remains to be seen.

There are still lots of open-ended questions related to transcriptional responses to glucocorticoids that will keep researchers busy for decades to come. For example, ChIP-seq experiments have uncovered thousands of GR binding sites and although regulated genes tend to have more GR binding sites in their vicinity, there are plenty of genes that are not regulated despite having a GR binding site nearby. This raises the question: What distinguishes a productive GR binding site (resulting in the regulation of associated genes) from ones where nearby genes are not regulated? A major complication in answering this question is that binding sites are assigned to a gene based on proximity along the linear DNA chain and not based on established functional connections between binding sites and genes. Some of these binding sites are located 100 s of kb away from the TSS and therefore could just as well be connected to other genes that are perhaps closer when the three dimensional organization of the nucleus is taken into account. Recently developed techniques to edit the genome like zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the CRISPR/Cas9 RNA-guided system provide the opportunity to disrupt genomic binding sites of GR and thereby to determine functional connections between binding sites and regulated genes [128–130]. Another approach to link binding sites to genes is to systematically determine the physical contacts between GR binding sites and TSSs of genes. Such long-range looping interactions can be identified with the use of chromatin conformation capture (3C)-based techniques and have shown a clear correlation between long-range contacts and transcriptional regulation by transcriptional regulatory factors [131, 132]. A final challenge is to understand how the integration of various inputs warrants that the right genes are expressed at the correct level in response to glucocorticoids. Many of these inputs that modulate the transcriptional responses have been identified including receptor isoform, post-translational modification state, ligand and interaction with other biological macromolecules including proteins and DNA. Likely however, additional inputs exist. For example, the role of the non-coding RNA universe is still largely unexplored and studies with ER have shown that so called enhancer RNAs (eRNAs, see Fig. 3.5) produced at ER binding sites are required for long-range looping and the transcriptional regulation of ER target genes [133]. Ultimately, a detailed knowledge of the signaling inputs and how they are integrated at individual genes will yield a greater understanding of the heterogeneity in GR signaling in health and disease and may one day improve the therapeutic use of glucocorticoids in the clinic.

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