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Authors

Chen, Sheng-Hong
Masuno, Kiriko
Cooper, Samantha
et al.

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Incoherent feed-forward regulatory logic underpinning glucocorticoid receptor action

Sheng-hong Chen[1], Kiriko Masuno, Samantha B. Cooper, and Keith R. Yamamoto[2]

Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA 94143

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The complexity and specificity of metazoan transcription are determined by combinatorial control of the composition and activity of regulatory complexes. To investigate the basis of this specificity, we focused on the glucocorticoid receptor (GR), a single regulatory factor that integrates multiple signals to give rise to many distinct patterns of expression. We measured the expression of a set of genes, each directly GR-regulated, but by different mechanisms in two cell lines. We varied ligand (dose, chemistry, and duration of treatment), GR (expression level and functionality), and a non-GR regulatory factor that commonly interacts with GR. Our study revealed distinct expression patterns within this set of genes, but all could be modeled by an incoherent feed-forward regulatory logic. Cellular signals, operating on GR and other factors within regulatory complexes, may define and modulate the kinetics and strength of the activating or inhibitory paths of the regulatory logic. Thus, characterizing systems behavior by perturbing single or multiple signals can reveal general principles of regulation, providing an approach to the dissection and deconvolution of combinatorial control.

steroid hormone receptor | transcription network motif | transcriptional regulation | cell type-specific transcription

Gene transcription in metazoans orchestrates spatial and temporal programs during development and governs cell and tissue specificity with responses to physiologic and external cues throughout lifespan. Regulation of gene transcription is conferred by an elaboration of the original paradigm enunciated by Jacob and Monod (1) in which regulatory factors bind to specific sites close to a promoter to activate or repress RNA production. In metazoans, the regulatory factors are large, multicomponent complexes composed of distinct combinations of regulatory proteins, some bound to specific genomic sites remote from and/or proximal to promoters, others associated with these bound components via protein–protein interactions. Because these regulatory proteins are typically broadly expressed, their mixed assembly into unique regulatory complexes is likely determined by combinatorial principles (2–5) that govern a cascade of context-specific interactions among regulatory factors and DNA elements.

One approach to investigate combinational control is to assess the transcriptional consequences of perturbing cellular signals that affect the functions of regulatory factors. The glucocorticoid receptor (GR) provides an elegant focal point for such studies because it has been shown to receive and integrate multiple cellular signals and to oversee cell-specific gene networks (6, 7) for organismal development, stress responses, and metabolic homeostasis (8–10). In addition to the effects of ligand dosage, chemistry, and duration of treatment (11, 12), other GR signals include genomic glucocorticoid response elements (GREs), which typically comprise GR binding sequences (GBSs) as well as sites for non-GR transcriptional regulatory factors, GR-interacting regulatory and coregulatory factors, and posttranslational modifications of GR.

At least two of the signals, ligands and GBSs, have been shown to alter receptor conformation and to modulate its association with non-DNA-binding coregulators or other features of its activity that determine the specificity of GR-mediated transcription (13–15). The effects of non-GR transcriptional regulatory factors on GR conformation have been less explored, but they strongly affect GR function. In certain gene and cell contexts, for example, the relative c-Jun content of dimeric AP-1 factors governs whether GR activates or represses transcription (16).

Whole-genome analyses of gene expression and GR occupancy (17–19) may enable us to infer and search for general rules that govern specificity or regulatory logic underlying dynamic receptor function, at least at subsets of responsive genes. Thus, we set out to identify a set of GR-regulated genes that were likely to be sensitive to shifts in signals, to examine the consequences within that system of altering signaling contexts, and to assess whether we could uncover general patterns of systems behavior, deconvolute some of the signaling effects on GR, and infer a regulatory logic that could account for GR activity and specificity. In this study, we examined transcriptional dynamics at a set of GR-regulated genes in two different cell lines, by altering ligand dose, chemistry, duration of treatment, the level of c-Jun, and level and functionality of GR itself.

Results

Identification and Characterization of Differentially Regulated Genes. We set out to investigate and identify differentially regulated genes (DRGs), which are GR-regulated genes that are transcriptionally activated in one cell type and repressed in another cell type upon the same hormonal treatment. Distinct from other GR targets that are regulated only in one cell type but not another, DRGs appear to be "poised" for building cell-specific regulatory complexes at response elements, and thus might be especially sensitive to shifts in signals and therefore reveal some general rules of combinatorial specificity.

Using expression microarrays, we previously examined changes in transcriptional profiles after a 3-h treatment of 10^{-6} M dexamethasone (dex) in two human cell lines: A549 lung carcinoma and U2OS osteosarcoma stably expressing GR, known as U2OS-hGR. There were 31 putative DRGs identified out of the 2,268 and 1,731 dex-regulated genes in A549 and U2OS-hGR cell lines, respectively.

We measured by RT-qPCR the changes in the mRNA level of putative DRGs during a 24-h time course at 10^{-6} M dex in A549 and U2OS-hGR cells (Dataset S1 gives expression microarray data). As shown in Fig. 1*A*, we studied four DRGs that were exclusively activated or repressed at all time points. Whereas AREG mRNA was up-regulated in U2OS-hGR and down-regulated in A549 cells, the same treatment induced the opposite effects on the other three DRGs, ANKRD1, ID1, and EMP1 (Fig. 1*A*).

We then assessed whether the changes in DRG mRNA were sensitive to GR level by knocking down GR expression using siRNA (Fig. 1*B*) and examining the dex-dependent changes in the DRG mRNA levels (Fig. 1 *C* and *D*). In each cell line, GR-specific siRNA knocked down GR protein accumulation to less than 5% of control levels (Fig. 1*B*).

Author contributions: S.-h.C. and K.R.Y. designed research; S.-h.C. and K.M. performed research; S.-h.C., S.B.C., and K.R.Y. analyzed data; and S.-h.C. and K.R.Y. wrote the paper.

The authors declare no conflict of interest.

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[1]Present address: Department of Systems Biology, Harvard Medical School, Boston, MA 02115.

[2]To whom correspondence should be addressed. E-mail: yamamoto@cmp.ucsf.edu.

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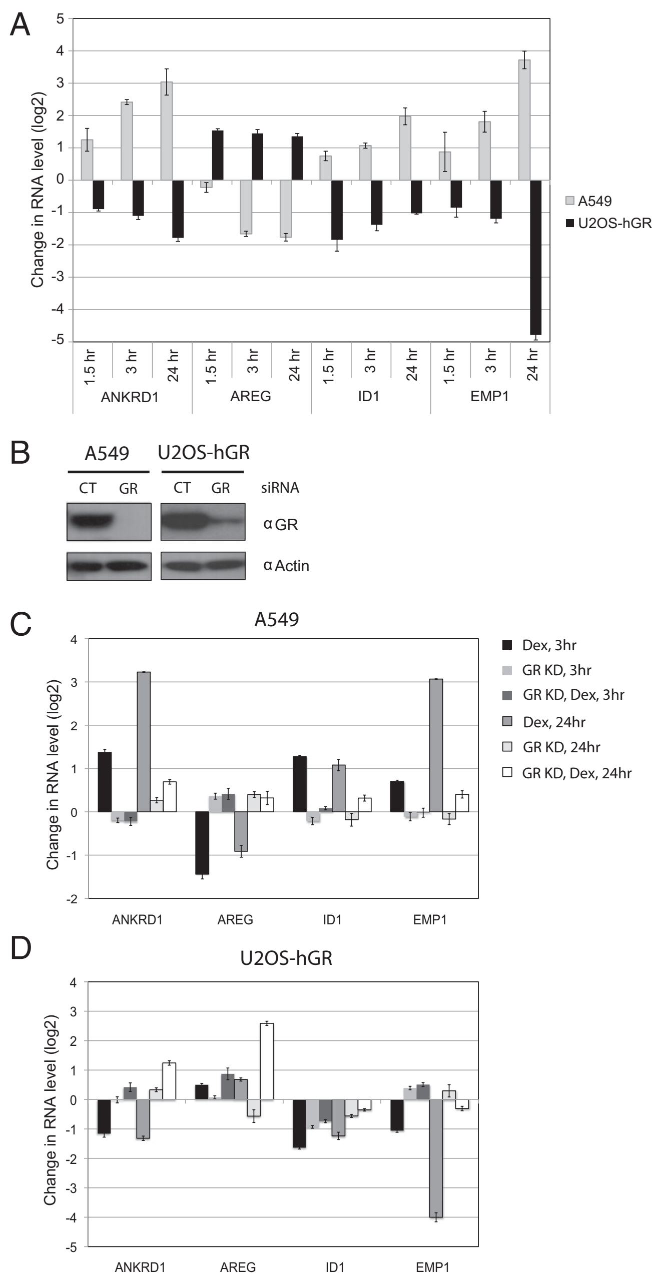

Fig. 1. Identification and characterization of DRGs. (*A*) Dexamethasone-stimulated changes in mRNA levels of DRGs. Cells were treated with vehicle [ethanol (EtOH)] or 10^{-6} M dex for 1.5, 3, or 24 h. Data are plotted in a log2 scale relative to EtOH. Data represent the mean ± SD, $n = 3$. (*B*) Immunoblots demonstrating the knockdown of GR. (*C* and *D*) Dexamethasone-stimulated changes in mRNA levels of DRGs after GR knockdown. Cells were transfected with either scrambled siRNA or siRNA specifically targeting GR transcripts, followed by treatment with EtOH or 10^{-6} M dex for 3 or 24 h. Transfection of two different scrambled siRNA sets showed similar results. Data are plotted in a log2 scale relative to EtOH. Data represent the mean ± SD, $n = 3$.

All four DRGs in A549 cells showed attenuation in dex-dependent transcriptional responses at both 3 and 24 h (Fig. 1*C*). In U2OS-hGR cells, however, we observed unexpected transcriptional changes in ANKRD1 and AREG (Fig. 1*D*). ANKRD1, initially identified to be repressed upon dex treatment, was activated after GR knockdown. AREG, initially identified to be activated upon dex treatment, was up-regulated to an even higher level when GR was knocked down (Fig. 1*D*). Thus, low levels of GR appeared to up-regulate both ANKRD1 and AREG mRNA levels, either changing from repression to activation (ANKRD1) or from a low to a higher magnitude of activation (AREG).

Effects of Ligand Dose and Duration of Treatment on DRG mRNA Levels. The results in Fig. 1*D* were counterintuitive, indicating elevated ANKRD1 and AREG induction upon GR knockdown. To interrogate this finding further, we tested two other ways to manipulate the cumulative level of GR activity, varying ligand dose and duration of treatment.

Fig. 2 *A*–*D* shows dose–response analyses of DRGs in both A549 and U2OS-hGR cells. In U2OS-hGR cells, both ANKRD1 and AREG mRNA levels increased as dex concentration increased from 10^{-11} M to 10^{-9} M. Remarkably, at dex concentrations $>10^{-9}$ M, ANKRD1 mRNA levels decreased sharply, whereas AREG mRNA declined modestly but significantly (solid lines in Fig. 2 *A* and *B*). Importantly, these decreases were maintained when cellular translation was inhibited by cycloheximide pretreatment, suggesting that the complex pattern of ANKRD1 mRNA production is not an indirect secondary effect of a GR-induced inhibitory protein.

In contrast to ANKRD1 and AREG in U2OS-hGR cells, the dose–response curves of those genes in A549 cells and those of ID1 and EMP1 in both cell lines appeared to be monotonic across the tested range of dex concentrations (solid lines in Fig. 2 *C* and *D* and dashed lines in Fig. 2 *A*–*D*).

We then measured DRG mRNA levels at different doses and durations of dex treatment in U2OS-hGR cells. Across a three-orders-of-magnitude variation in dex dosage and a 24-h time course (Fig. 2 *E*–*H*), we found that ANKRD1 mRNA levels increased at dex concentrations $<3 \times 10^{-10}$ M and durations of <3 h and then declined at dex concentrations $>3 \times 10^{-10}$ M and for times >3 h. That is, ANKRD1 mRNA production is initially activated and then repressed (here meant to include both deactivation and active repression) as the cumulative level of GR activity is increased across a range of ligand doses and durations of treatment.

Remarkably, all four DRGs displayed this idiosyncratic response to increasing levels of GR activity, although the patterns in detail were gene-specific. Notably, ID1 and EMP1, neither of which showed increases in their mRNA levels in dose–response curves (Fig. 2 *C* and *D*), showed mild but consistent increases at shorter durations and lower concentrations of dex treatment (Fig. 2 *G* and *H*; values for Fig. 2 *E*–*H* are shown in Dataset S2).

Effects of Ligand Chemistry on DRG Transcription in U2OS-hGR Cells. Alterations in ligand chemistry provide another way to modulate, quantitatively or qualitatively, GR activity. We therefore compared the effects of five GR ligands that differ in structure and GR binding affinities (dex > RU486 > prednisolone > cortisol > corticosterone) (20). With each ligand except RU486, the patterns of DRG responses were reminiscent of those seen with other methods for varying levels of GR activity. Thus, ANKRD1 transcription was first activated, then repressed (Fig. 3*A*), in a ligand-specific manner that roughly correlated with their GR binding affinities; higher-affinity ligands induced stronger activation at low concentrations and stronger repression at high concentrations.

In the case of AREG, most tested ligands yielded dose–response patterns similar to those seen with ANKRD1, except prednisolone induced the highest level of expression, and cortisol and corticosterone failed to repress even at the highest concentrations tested (Fig. 3*B*). In contrast, ID1 and EMP1 were both repressed by all ligands, and the extent of repression correlated roughly with GR binding affinities (Fig. 3 *C* and *D*).

Distinct from the other ligands, RU486 did not trigger substantial repression of ANKRD1 and AREG (except perhaps at the highest concentration), nor did it induce strong repression of ID1 and EMP1 despite its high affinity binding to GR.

Interaction Between c-Jun and Glucocorticoid Signaling. The AP-1 family of regulatory factors commonly interacts with GR functionally; in some cases the subunit composition of AP-1 dimeric

SYSTEMS BIOLOGY

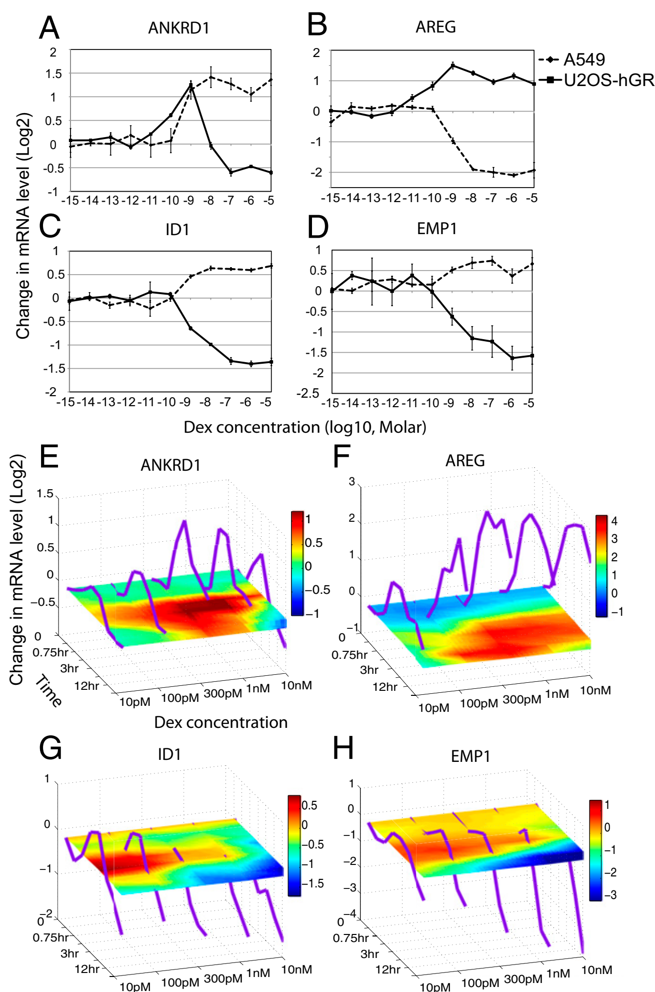

Fig. 2. Dose–response curves and dose and temporal kinetics of DRGs in U2OS-hGR cells. (*A*–*D*) Dexamethasone-stimulated changes in mRNA levels of DRGs. Cells were treated with EtOH or dex (10^{-15} M to 10^{-5} M) for 3 h. Data are plotted in a log2 scale relative to EtOH. Data represent the mean ± SD, n = 3. (*E*–*H*) Dexamethasone-stimulated changes in mRNA levels of DRGs. Cells were treated with EtOH or dex (10 pM to 10 nM) for 0, 0.375, 0.75, 1.5, 3, 6, 12, or 24 h. Data are plotted in a log2 scale relative to EtOH. Data represent the mean, and Dataset S2 gives the mean and SD, n = 3.

complexes is a determinant of activation or repression by GR (16). Therefore, we tested whether expression levels of the c-Jun subunit of AP-1 differentially affected GR-mediated regulation of the DRGs. Knocking down c-Jun expression with siRNA (Fig. 4*A*), we examined the dex dose–response curves of the DRGs (Fig. 4 *B*–*I*). In both A549 and U2OS-hGR cells, siRNA knockdown of c-Jun expression was >90% effective (Fig. 4*A*), with no accompanying effect on GR expression (Fig. 4*A*).

As shown in Fig. 4 *B*–*I*, c-Jun displayed three distinct patterns of dose-, gene-, and cell-specific effects on GR regulation of the DRGs. First, c-Jun and glucocorticoid signaling interacted synergistically at AREG (Fig. 4*C*) and EMP1 (Fig. 4*E*) in A549 cells; c-Jun knockdown rendered both genes less responsive to dex $>10^{-10}$ M. In other contexts, c-Jun and glucocorticoid signaling interacted antagonistically, as implied by stronger dex-dependent transcriptional changes after c-Jun knockdown. These interactions occurred at ANKRD1 at $>10^{-10}$ M dex (Fig. 4*F*), at AREG at 10^{-10} M to 10^{-9} M dex (Fig. 4*G*), and at EMP1 at 10^{-9} M dex (Fig. 4*I*) in U2OS-hGR cells and at ID1 at $>10^{-10}$ M dex in both cell types (Fig. 4 *D* and *H*). Finally, c-Jun knockdown had no effect on GR-regulated expression of ANKRD1 in A549 cells (Fig. 4*B*).

Regulation of ANKRD1 Repression. ANKRD1 expression at low and high concentrations of dex could be viewed as an example of differential GR-mediated regulation within a single cell type (Fig. 2*A*). We first tested whether the apparent ANKRD1 repression might instead reflect reduced levels of activation triggered by a dex-driven decline of GR levels in U2OS-hGR cells. As shown in Fig. 5*A*, however, GR protein levels were unaffected by dex.

We next examined the effects of various GR mutations on ANKRD1 repression by developing U2OS cell lines stably expressing GR derivatives bearing mutations affecting GR-regulated activation or repression, including P474R/A475S (21), G439E/S440G/V443G (22), S425G/E427G (23), A458T (23), and E755R (7). We found that only the A458T mutation, which decreases the cooperativity of GR dimerization and increases dissociation rates at GR binding sequences in vitro (24), altered the dex dose-dependent repression of ANKRD1. Indeed, this mutation virtually abolished repression but had little or no effect on activation (Fig. 5*B*); as with wild-type GR, expression of GR A458T protein was unaffected by dex treatment (Fig. 5*A*).

We then monitored GR occupancy after 45 min of 10^{-9} M and 10^{-6} M dex treatment at candidate GREs around the ANKRD1 gene (defined here as GR binding regions within ±20 kb of the transcription start site). ChIP-seq experiments have identified four such regions, E1–E4 (12 and Fig. 5*C*). In U2OS-hGR (A458T) and A549 cell lines, GR occupancy at the E1, E2, and E3 sites was elevated slightly, but significantly, at 10^{-6} M dex, whereas GR occupancy declined at these three sites in U2OS-hGR cells. At the E4 site, GR occupancy increased in all three cell lines at the high dex concentration (Fig. 5*D*). The results at E1–E3 imply that cooperative GR binding might be involved in repression of ANKRD1 at 10^{-6} M dex.

Discussion

A model for the complexity and specificity of metazoan transcriptional regulation is combinatorial control (2–5), in which regulatory factors assemble at response elements in distinct permutations to form response element-specific multicomponent regulatory complexes. This combinatorial assembly reflects the integration of multiple signals and can involve dozens to hundreds of proteins; in this way, broadly expressed factors in different combinations can generate unique transcriptional outcomes. However, neither the precise determinants of specificity nor the regulatory logic underlying the dynamic behavior of transcription is well understood.

Here, we took a systems approach to investigate the specificity of GR-regulated transcription within a set of differentially regulated genes (DRGs). We monitored the consequences of altering—independently or in combination—some of the signals integrated by GR, including ligand dose, chemistry, duration of treatment, the level of c-Jun, and the level and functional surfaces of GR itself. We observed "pulse-like dynamics," that is, an initial GR-mediated activation followed by a delayed repression that were independent of translational feedback in DRGs in U2OS-hGR cells. We thus inferred an incoherent feed-forward regulatory logic underpinning DRG behavior.

Interestingly, different GR ligands altered the potency of both the activating and the inhibitory arms of this regulatory circuit, whereas c-Jun interacted with one or the other path, but not both. In the case of ANKRD1 in U2OS-hGR cells, cooperative binding of GR could selectively affect the inhibitory path. Thus, regulatory specificity could be defined by perturbations of subsets of the signals that together define the regulatory logic.

Regulatory Logic for DRG Transcription. Strikingly, we discovered inflection points in the GR-regulated behavior of ANKRD1, and systematic signal perturbations suggested that its transcription is sensitive to the levels of GR activity (Fig. 1*D*), influenced by concentrations of dex (Fig. 2*A*), duration of treatment (Fig. 2*E*), and other parameters. We speculate that a certain level of GR activity, as determined by ligand dose, duration of treatment, or GR expression, would trigger repression of ANKRD1 (Fig. 5*E*).

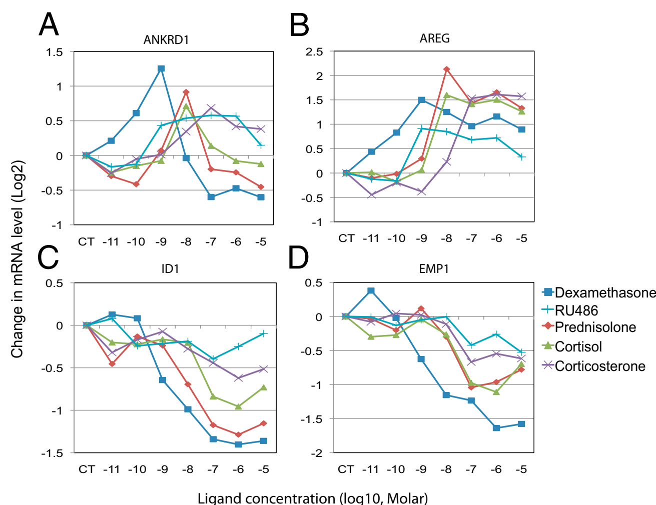

Fig. 3. Dose–response curves of DRGs induced by different ligands. (*A*–*D*) Dexamethasone-stimulated changes in mRNA levels of DRGs. Cells were treated with EtOH, RU486, prednisolone, cortisol, or corticosterone (10^{-11} M to 10^{-5} M) for 3 h. Data are plotted in a log2 scale relative to EtOH (labeled CT). Data represent the mean, and Dataset S3 gives mean and SD, *n* = 3.

Whereas the detailed expression patterns of the different DRGs were distinct, they clearly shared a general feature: activation at the onset of glucocorticoid signaling followed by repression (Fig. 2 *E*–*H*). This regulatory behavior is reminiscent of one of the eight "feed-forward loop" network motifs described in *E. coli* and yeast by Mangan and Alon (25). Specifically, the incoherent type-1 feed-forward loop (I1-FFL) produces pulse-like dynamics driven by an activator that operates both on a terminal target and an inhibitor of that target, resulting in an initial activation followed by a delayed repression of the terminal target (25, 26). Strikingly, the expression patterns of each of our DRGs could be shaped parsimoniously by the I1-FFL network motif (Fig. 5*E*).

In U2OS-hGR cells, the "activating arm" of the motif is initiated at low levels of GR activity, and the "inhibitory arm" is initiated when GR activity exceeds an inhibitory threshold (Fig. 5*E*). The molecular determinants of this threshold are unknown, but achieving the threshold does not seem to reflect induction of an inhibitory protein, because the regulatory pattern was unaffected by an inhibitor of protein synthesis.

Notably, repression of the DRGs was not observed in A549 cells at high dex concentrations. Because the level of GR expression is lower in A549 cells (about 20% of that seen in U2OS-hGR cells; Fig. S1), the inhibitory path may function relatively rarely in A549 cells. Indeed, repression of DRGs was compromised when GR expression was knocked down in U2OS-hGR cells (Fig. 1*D*). For example, the higher induction of ANKRD1 and AREG at 24 h could be due to the lack of inhibitory activity when GR level is low after knockdown. The level of GR activity might gradually increase over the 24-h period yet never reach the threshold for inhibition. Thus, cells might use the incoherent feed-forward loop differently as a function of different levels of GR activity.

Remarkably, pulse-like dynamics, previously observed in another signaling pathway (26), were observed in all four DRGs in U2OS-hGR cells. How these dynamics are achieved remains to be explored. Indeed, we do not know which step(s) of transcription is regulated by GR among the DRGs. Preliminary experiments with ANKRD1 upstream regulatory sequences yielded reporter transcripts with dynamics similar to ANKRD1 mRNA, suggesting that GR may regulate ANKRD1 transcriptional initiation. The physiological significance of the pulse-like dynamics is also unknown, but it is apparent that the I1-FFL network motif can produce precise and specific transcriptional control in a given context while also enabling flexibility—a shift in the direction of GR-mediated regulation in different cell-type or GR-activity contexts. Solving this "precision-flexibility paradox" is essential for achieving context-specific transcriptional regulation, and we show here that the switchlike behavior of the I1-FFL network motif provides a general way to address that paradox.

Whereas the I1-FFL motif likely governs transcription of other GR-regulated genes as well, there are GR-mediated expression patterns not readily explained by this regulatory logic. One example is the expression dynamics in which repression occurs at lower concentrations of hormone than activation (27). A GRE that confers this pattern of expression may specify the assembly of a regulatory complex that generates an incoherent type-3 feed-forward loop (25), where low levels of active GR initially repress target gene expression and then activate it when the level of GR increases through some threshold.

Thus, GR confers multiple classes of transcriptional dynamics (11), each perhaps fitted to a particular network motif (25, 28)

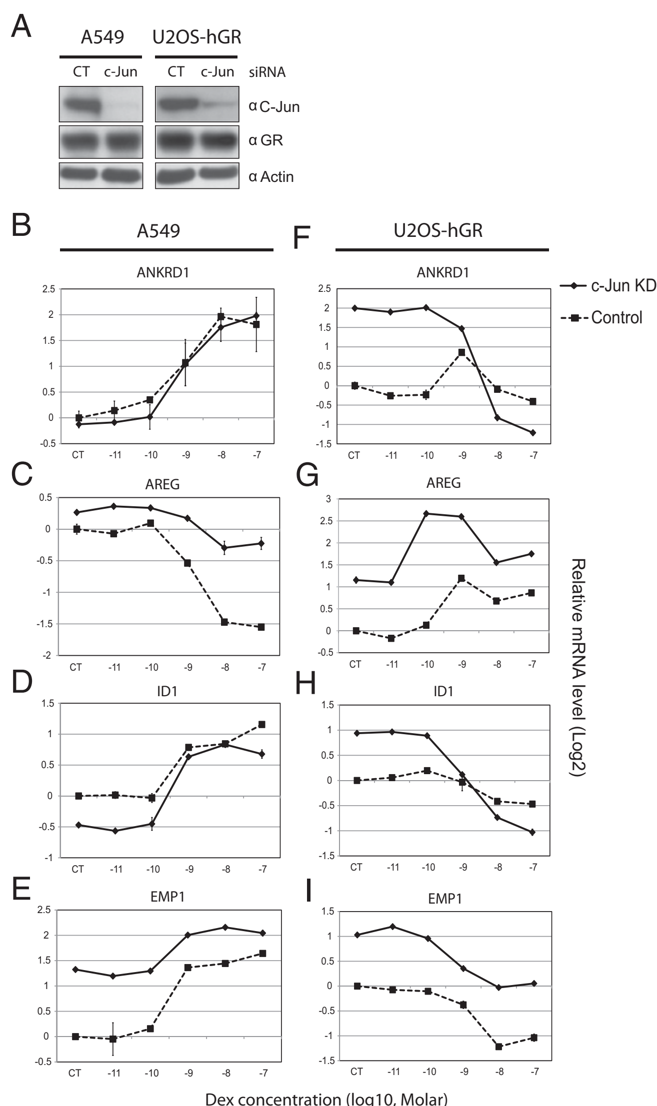

Fig. 4. C-Jun interacts with glucocorticoid signaling in a context-dependent manner. (*A*) Immunoblots demonstrating the knockdown of c-Jun. The loading controls are the immunoblots of actin from the same samples. (*B*–*I*) Dexamethasone-stimulated changes in mRNA levels of DRGs after c-Jun knockdown. Cells were transfected with either scrambled siRNA (control) or siRNA specifically targeting c-Jun transcripts, followed by treatment with EtOH or dex (10^{-11} M to 10^{-7} nM) for 3 h. Data are normalized with the level of *RPL19* in the control and plotted in a log2 scale relative to EtOH (labeled CT). Data represent the mean ± SD, *n* = 3.

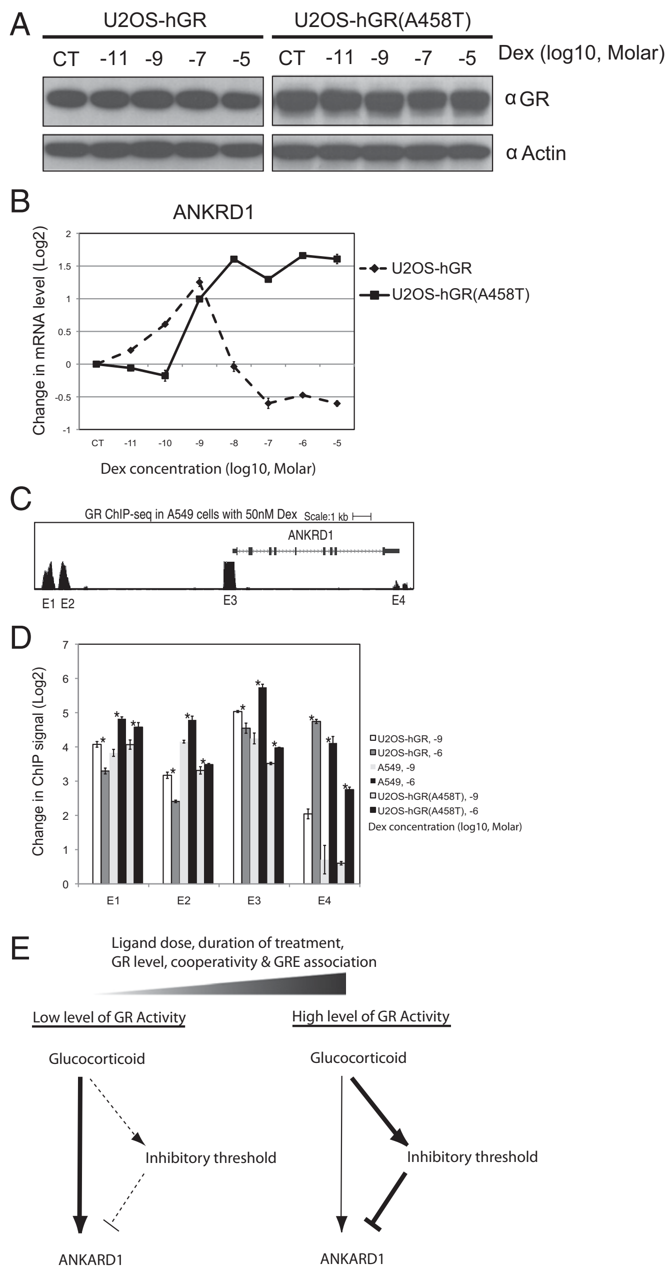

Fig. 5. Regulation of ANKRD1 repression. (*A*) Immunoblots demonstrating the level of GR did not change in either wild type or A548T mutant. Cells were treated with EtOH or dex (10^{-11} M to 10^{-5} M) for 1.5 h. Immunoblots at lower exposure showed similar relative GR intensities. (*B*) Dose–response curves of ANKRD1 in U2OS-hGR(A548T). U2OS-hGR(A548T) cells were treated with EtOH or dex (10^{-11} M to 10^{-5} M) for 3 h. Data are plotted in a log2 scale relative to EtOH (labeled CT) and represent the mean ± SD, $n = 3$. (*C*) ChIP-seq data of GR occupancies at ANKRD1 gene (12). (*D*) ChIP showing GR occupancy at E1–E4 sites around ANKRD1. A549, U2OS-hGR, and U2OS-hGR(A548T) cells were treated with EtOH, 10^{-9} M, or 10^{-6} M dex for 45 min. Data are plotted in a log2 scale relative to EtOH and represent the mean ± SD, $n = 3$. The GR occupancies are significantly different between 1-nM and 1-μM dex treatment at all sites and in all three cell lines (P value < 0.05 by Student's t test). (*E*) Model for the incoherent feed-forward loop control of ANKRD1 differential transcription.

that potentially provides a parsimonious solution to the precision-flexibility paradox.

Ligand Signaling. For the ligands tested other than RU486, the potency of activation and repression of the DRGs seemed generally to correlate with the relative affinities of the ligands for GR (Fig. 3 *A–D*). Previous studies showed that ligands with higher binding affinities commonly trigger higher fractional nuclear translocation of GR (13, 29) but slower GR mobility in the nucleus (20). Conceivably, an aggregate function of translocation efficiency, intranuclear mobility, and GRE occupancy might be a determinant of overall GR activity. In principle, manipulating ligand chemistry and binding affinities could modulate the initiation and intensity of the activating and inhibitory arms in the incoherent feed-forward loop.

Notably, RU486 displayed a distinct dose–response behavior compared with other ligands. RU486 induced ANKRD1 and AREG activation at very low concentrations (Fig. 3 *A* and *B*) and acted as a weak agonist for DRGs. In the case of ANKRD1, however, RU486 triggered weak if any repression (Fig. 3*A*), and for two repressed genes, ID1 and EMP1, RU486 similarly conferred weak repression (Fig. 3 *C* and *D*). Thus, despite its high affinity, RU486 establishes or maintains the inhibitory path poorly compared with the other ligands. Previous studies on ligand-induced GR coregulator binding suggest that RU486 binding triggers GR activities and conformations quite distinct from those provoked by other ligands examined here (15). Accordingly, RU486 may establish a relatively novel allosteric path that in turn creates a distinct route of signal integration for DRG transcriptional regulation.

c-Jun Signaling. Knockdown of c-Jun, a common GR collaborator, revealed dose-, cell-, and gene-specific interactions between c-Jun and glucocorticoid signaling: synergistic interactions in A549 cells and antagonistic interactions in U2OS-hGR cells. Conceivably, these interactions are regulated via cell-specific ratios of c-Jun relative to other AP-1 family members (16, 30).

As expected, the c-Jun–GR interaction patterns are strongly context-dependent. For example, the strongest antagonistic interaction occurs at 10^{-10} M dex for AREG, and at 10^{-9} M to 10^{-7} M dex for ANKRD1. Thus, cJun interacts independently and gene-specifically with the activating or inhibitory arms of the GR-mediated feed-forward loop. This relationship of c-Jun signaling to GR seems distinct from ligand-mediated signaling, where modulation of the activating and inhibitory paths are correlated.

Our results imply a dynamic association of c-Jun with certain GR regulatory complexes, which seems to depend on the level of GR activity and the structure and composition of GREs. Interestingly, each of the putative GREs of ANKRD1, E1-E4, contained mixtures of GR and AP-1 binding sequences, suggesting composite response elements conferring combinatorial control both by sequence-specific DNA binding of GR and AP-1 and by protein–protein interactions between them.

Whether c-Jun indeed acts directly at the putative GREs of DRGs or, rather, affects their activities indirectly is unknown. In fact, unequivocal assignment of putative GREs to specific target genes is a substantial challenge, as is dissection of the dynamic interactions of factors within the regulatory complexes. One approach might be to measure the activities of GRE-bearing reporters after integration at a common genomic "safe harbor" site, providing a bona fide isogenic setting for quantitative analysis (31). The interaction between c-Jun and glucocorticoid signaling is likely a complex function of ligand chemistry and dose, GRE specificity, and more. Dissection of these context-dependent interactions will illuminate the consequences of signal integration on the implementation of regulatory logic.

Effects of GR Functional Domains. Screening of mutations in GR functional surfaces for those that abolish ANKRD1 repression identified A458T, a dimerization interface mutant that reduces GR binding cooperativity and increases dissociation rates at GR binding sequences in vitro (24). Notably, we observed similar patterns of GR occupancy at the ANKRD1 E1–E3 sites in U2OS-hGR(A548T) and A549 cell lines at low and high doses of dex treatment (Fig. 5*D*). Neither cell line showed ANKRD1 repression at high concentrations of dex. Thus, cooperative

binding of GR might be critical for repression of ANKRD1 at higher concentrations of ligand and responsible for dose-specific changes in GR occupancy at putative GREs of ANKRD1. In contrast, activation of ANKRD1 seems independent of the A548-containing surface of GR (Fig. 5*B*).

The regulatory patterns characteristic of our DRGs are distinct from those of GREs at which GR confers repression at low levels of GR activity by tethering through protein–protein interactions to other transcriptional regulatory factors (27). We speculate that repression conferred by our DRG GREs may reflect differential competition for limiting GR coregulators. That is, GREs associated with highly expressed genes might compete for coregulators at higher concentrations of dex through a process dependent on GR dimerization. For weakly expressed genes such as ANKRD1, however, co-occupancy by factors essential for GR-mediated activation might fail to occur in the absence of certain limiting coregulators, resulting in repression at high levels of GR activity. Interestingly, ANKRD1 activation does not seem to require highly cooperative binding of GR (Fig. 5*B*), consistent with our proposed model (Fig. 5*E*).

Mangan and Alon (25) proposed FFL motifs as interlocked transcription circuits; the I1-FFL, for example, posits a transcriptional activator that induces both a terminal target gene and a second target, which represses the terminal target. Because multiple variables that affect levels of GR activity seem to affect differentially the activation and inhibitory arms of the I1-FFL described here, we suggest that the operating components of this network motif may reside within the GR-containing regulatory complexes at the DRG GREs, with the two arms governed by signaling rather than by transcription. Notably, Alon (32) pointed out that signal transduction networks may also display network motifs. This regulatory logic provides a conceptual model to further dissect the integration processes of a wide range of signals and led us to identify a repression activity dependent on high levels of GR activity and the GR dimerization interface. Addressing the challenges raised by our study will continue to provide new insights into the general principles and regulatory logic for the combinational control of gene transcription.

Materials and Methods

SI Materials and Methods includes information on plasmids, chemicals and proteins, cell culture, cell line development, RNA isolation, RT-qPCR, siRNA knockdown, and ChIP. Constructs produced in the K.R.Y. laboratory are available on request. Please contact Teresita Bernal, tbernal@cmp.ucsf.edu.

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1. Jacob F, Monod J (1961) Genetic regulatory mechanisms in the synthesis of proteins. *J Mol Biol* 3(3):318–356.
2. Gierer A (1974) Molecular models and combinatorial principles in cell differentiation and morphogenesis. *Cold Spring Harb Symp Quant Biol* 38:951–961.
3. Yamamoto KR (1989) A conceptual view of transcriptional regulation. *Integr Comp Biol* 29(2):537–547.
4. Levine M, Tjian R (2003) Transcription regulation and animal diversity. *Nature* 424 (6945):147–151.
5. Britten RJ, Davidson EH (1969) Gene regulation for higher cells: a theory. *Science* 165 (3891):349–357.
6. Wang J-C, et al. (2004) Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. *Proc Natl Acad Sci USA* 101(44):15603–15608.
7. Rogatsky I, et al. (2003) Target-specific utilization of transcriptional regulatory surfaces by the glucocorticoid receptor. *Proc Natl Acad Sci USA* 100(24):13845–13850.
8. Jobe AH, Ikegami M (2000) Lung development and function in preterm infants in the surfactant treatment era. *Annu Rev Physiol* 62:825–846.
9. Yamamoto KR, Darimont BD, Wagner RL, Iñiguez-Lluhí JA (1998) Building transcriptional regulatory complexes: Signals and surfaces. *Cold Spring Harb Symp Quant Biol* 63:587–598.
10. Sorrells SF, Caso JR, Munhoz CD, Sapolsky RM (2009) The stressed CNS: When glucocorticoids aggravate inflammation. *Neuron* 64(1):33–39.
11. John S, et al. (2009) Kinetic complexity of the global response to glucocorticoid receptor action. *Endocrinology* 150(4):1766–1774.
12. Reddy TE, et al. (2009) Genomic determination of the glucocorticoid response reveals unexpected mechanisms of gene regulation. *Genome Res* 19(12):2163–2171.
13. Hadley KE, Louw A, Hapgood JP (2011) Differential nuclear localisation and promoter occupancy play a role in glucocorticoid receptor ligand-specific transcriptional responses. *Steroids* 76(10–11):1176–1184.
14. Freedman ND, Yamamoto KR (2004) Importin 7 and importin alpha/importin beta are nuclear import receptors for the glucocorticoid receptor. *Mol Biol Cell* 15(5):2276–2286.
15. Ronacher K, et al. (2009) Ligand-selective transactivation and transrepression via the glucocorticoid receptor: Role of cofactor interaction. *Mol Cell Endocrinol* 299(2): 219–231.
16. Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR (1990) Transcription factor interactions: Selectors of positive or negative regulation from a single DNA element. *Science* 249(4974):1266–1272.
17. Farnham PJ (2009) Insights from genomic profiling of transcription factors. *Nat Rev Genet* 10(9):605–616.
18. Garber M, Grabherr MG, Guttman M, Trapnell C (2011) Computational methods for transcriptome annotation and quantification using RNA-seq. *Nat Methods* 8(6): 469–477.
19. Pepke S, Wold B, Mortazavi A (2009) Computation for ChIP-seq and RNA-seq studies. *Nat Methods* 6(11, Suppl):S22–S32.
20. Schaaf MJM, Lewis-Tuffin LJ, Cidlowski JA (2005) Ligand-selective targeting of the glucocorticoid receptor to nuclear subdomains is associated with decreased receptor mobility. *Mol Endocrinol* 19(6):1501–1515.
21. Yang-Yen HF, et al. (1990) Transcriptional interference between c-Jun and the glucocorticoid receptor: Mutual inhibition of DNA binding due to direct protein-protein interaction. *Cell* 62(6):1205–1215.
22. Schüle R, et al. (1990) Functional antagonism between oncoprotein c-Jun and the glucocorticoid receptor. *Cell* 62(6):1217–1226.
23. Heck S, et al. (1994) A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. *EMBO J* 13(17): 4087–4095.
24. Watson LC, et al. The glucocorticoid receptor dimer interface allosterically transmits sequence-specific DNA signals. *NSMB*, in press.
25. Mangan S, Alon U (2003) Structure and function of the feed-forward loop network motif. *Proc Natl Acad Sci USA* 100(21):11980–11985.
26. Amit I, et al. (2007) A module of negative feedback regulators defines growth factor signaling. *Nat Genet* 39(4):503–512.
27. Jonat C, et al. (1990) Antitumor promotion and antiinflammation: Down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. *Cell* 62(6):1189–1204.
28. Mangan S, Itzkovitz S, Zaslaver A, Alon U (2006) The incoherent feed-forward loop accelerates the response-time of the gal system of Escherichia coli. *J Mol Biol* 356(5): 1073–1081.
29. Savory JG, et al. (1999) Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. *Mol Cell Biol* 19(2):1025–1037.
30. Kerppola TK, Luk D, Curran T (1993) Fos is a preferential target of glucocorticoid receptor inhibition of AP-1 activity in vitro. *Mol Cell Biol* 13(6):3782–3791.
31. DeKelver RC, et al. (2010) Functional genomics, proteomics, and regulatory DNA analysis in isogenic settings using zinc finger nuclease-driven transgenesis into a safe harbor locus in the human genome. *Genome Res* 20(8):1133–1142.
32. Alon U (2007) Network motifs: Theory and experimental approaches. *Nat Rev Genet* 8(6):450–461.