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Conventional and pioneer modes of glucocorticoid receptor interaction with enhancer chromatin *in vivo*

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ABSTRACT

Glucocorticoid hormone plays a major role in metabolism and disease. The hormone-bound glucocorticoid receptor (GR) binds to a specific set of enhancers in different cell types, resulting in unique patterns of gene expression. We have addressed the role of chromatin structure in GR binding by mapping nucleosome positions in mouse adenocarcinoma cells. Before hormone treatment, GR-enhancers exist in one of three chromatin states: (i) Nucleosome-depleted enhancers that are DNase I-hypersensitive, associated with the Brg1 chromatin remodeler and flanked by nucleosomes incorporating histone H2A.Z. (ii) Nucleosomal enhancers that are DNase I-hypersensitive, marked by H2A.Z and associated with Brg1. (iii) Nucleosomal enhancers that are inaccessible to DNase I, incorporate little or no H2A.Z and lack Brg1. Hormone-induced GR binding results in nucleosome shifts at all types of GR-enhancer, coinciding with increased recruitment of Brg1. We propose that nucleosome-depleted GR-enhancers are formed and maintained by other transcription factors which recruit Brg1 whereas, at nucleosomal enhancers, GR behaves like a pioneer factor, interacting with nucleosomal sites and recruiting Brg1 to remodel the chromatin.

INTRODUCTION

The mechanism by which glucocorticoid hormone controls gene expression is central to an understanding of its role in human metabolism and disease. When the hormone enters a cell, it binds to the glucocorticoid receptor (GR), inducing translocation from the cytoplasm to the nucleus, where it binds to glucocorticoid response elements (GREs) to activate or repress target genes. GR binds different GREs in each type of cell, resulting in regulation of a different set of target genes. How the cell determines which GREs should be bound by GR to initiate the required change in gene expression pattern is an important question. Both chromatin structure and DNA methylation play a major role in this regulation (1–3).

The structural subunit of chromatin is the nucleosome, which contains ∼147 bp of DNA wrapped almost twice around a central octamer composed of two molecules each of the four core histones: H2A, H2B, H3 and H4 (4). Generally, the compact structure of the nucleosome strongly inhibits the binding of transcription factors to their sites. To facilitate access for transcription factors, active promoters are often depleted of nucleosomes, forming 'nucleosome-depleted regions' (NDRs), which are flanked by relatively unstable nucleosomes containing the histone variants H2A.Z and H3.3 (5). Similarly, enhancers, which are regulatory elements typically located far from their target promoters, are often associated with much higher accessibility to nucleases such as DNase I, nucleosomes containing H2A.Z and H3.3, and specific histone modifications (6–9). However, unlike most transcription factors and similar to pioneer factors (10), GR binds strongly to a GRE in a nucleosome *in vitro*, although its affinity for a histone-free GRE is higher (11–13). It is therefore unclear whether a nucleosome can block GR binding *in vivo*.

The majority of cell-specific GR-enhancers are hypersensitive to DNase I even before the addition of hormone; they appear to be primed or 'pre-programmed' for GR binding. Another subset of enhancers are initially insensitive to DNase I and appear to be actively opened by GR ('*de novo*' sites) (2,3,14). The changes in chromatin structure which underlie DNase I accessibility remain unclear (15,16). In the simplest model, DNase I accessibility at enhancers indi-

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cates the presence of a nucleosome-depleted region, where the DNA is much less protected from digestion. If true, responsive GR-enhancers (those that bind GR in a specific cell type) should be DNase I-accessible and nucleosome-depleted, whereas unresponsive GR-enhancers (those that do not bind GR in a specific cell type) should be inaccessible and nucleosomal.

Some evidence for this model derives from nucleosome mapping in human acute lymphoblastic leukemia (ALL) cells (17). These authors argue that the average GR-enhancer is located in an NDR that is unaffected by hormone. However, analogous studies of other steroid hormone receptors, such as the androgen receptor (AR), progesterone receptor (PR) and estrogen receptor (ER), which respond to hormone in similar ways to GR, suggest alternative models for the interaction of the receptor with chromatin. In prostate cancer cells, androgen response elements (AREs) are initially nucleosomal but partly depleted of nucleosomes in response to hormone (18,19). In human breast cancer cells, progesterone response elements (PREs) are hypersensitive to DNase I and yet have very high nucleosome occupancy before progesterone treatment (20). Progesterone induces remodeling of the PRE-nucleosome, resulting in the loss of H2A-H2B dimers, although nucleosome occupancy remains very high. A related model has been proposed for the binding of ER (21). Thus, binding to response elements located within NDRs (17) apparently distinguishes GR from AR, PR and ER, which interact with nucleosomal response elements.

To improve our understanding of the interaction of GR with chromatin, we have examined the relationship between hormone-induced GR binding, nucleosome occupancy, H2A.Z incorporation and presence of the Brg1 ATP-dependent chromatin remodeling complex in mouse mammary adenocarcinoma 3134 cells. We mapped nucleosomes genome-wide by paired-end sequencing of nucleosomal DNA obtained by micrococcal nuclease digestion (MNase-seq). We obtained sufficiently high resolution to resolve events occurring at GREs in chromatin. We mapped H2A.Z incorporation sites and Brg1 binding in chromatin using ChIP-seq. We show that, before hormone treatment, most GR-responsive enhancers are nucleosomal, with a minority located within a pre-existing NDR. After hormone-induced GR binding, Brg1 is recruited to all GR-responsive enhancers, correlating with nucleosome shifts away from the GR-site.

MATERIALS AND METHODS

Cell lines

The cell lines used for chromatin immunoprecipitations (ChIP) of Flag-tagged H2A.Z were developed from the mouse 7110 cell line (22,23). Briefly, the 7110 cell line is a 3134 daughter line that contains an integrated 'Tet-off' gene expression system using a tetracycline-binding/VP16 fusion protein as a trans-activator. The H2A.Z gene was inserted into the Rosa26 gene trap locus using zinc-finger nucleases (Sangamo) under puromycin selection and Tet expression control. H2A.Z expression was induced by removing Tet from cells for 24 h and compared to cells that remained on Tet. After 24 h, cells without Tet were treated with vehicle or 600 nM corticosterone for 60 min. In wash-out experiments, cells were treated for 60 min with hormone, washed to remove the hormone and collected 30 min later, or given 90 min of constant hormone treatment prior to harvest.

MNase digests

Mouse 3134 cells were cultured, treated with vehicle or 100 nM dexamethasone for 1 h prior to harvest, and nuclei were prepared as described (23), except for the following modifications. Prior to MNase digestion, nuclei were washed in Nuclei Suspension Buffer (25% glycerol, 5 mM Mg-acetate, 5 mM HEPES pH 7.3, 0.08 mM EDTA, 0.5 mM spermidine, 1 mM DTT) and resuspended in MNase Digestion Buffer (10 mM Tris–HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 mM spermidine) at 75 million nuclei/ml and 400 U/ml MNase (Worthington). Nuclei were digested in 400 ul aliquots for 4–7 min at 37°C with gentle agitation; the reaction was terminated with Stop Buffer (500 mM NaCl (140 mM final), 50 mM EDTA (14 mM final), 20 mM EGTA (5.6 mM final), 3.6% SDS (1% final)) to yield ∼90% mono-nucleosomes (Supplementary Figure S1). The digests were treated with RNase (Life Technologies) for 30 min, then proteinase K (Ambion) for another 30 min. Samples were extracted twice with phenol/chloroform and ethanol-precipitated. DNA pellets were washed twice with 70% ethanol, dried and dissolved in water. Nicks in MNase-digested DNA were repaired using the PreCR repair mix (NEB) and the DNA was purified using a PCR purification kit (Life Technologies).

Paired-end sequencing

Sequencing libraries were either prepared as described (24,25) from the entire MNase digest (Experiment 1) or mono-nucleosomal DNA was isolated by electroelution from a 6% polyacrylamide gel (Experiment 2). Paired-end sequencing was performed using the Illumina High-Seq and/or Illumina NextSeq platforms.

ChIP-seq

Cells were cross-linked for 10 min in 1% formaldehyde at room temperature and subsequently quenched with 150 mM glycine for 10 min. Fixed cells were sonicated in a cold water bath (Diagenode) for a total of 120 s. Each ChIP reaction contained 1200 μg of pre-cleared soluble chromatin and immunoprecipitated overnight with anti-Flag antibody (Sigma F1804) conjugated to anti-mouse IgG magnetic beads (Life Technologies). Precipitated DNA was purified according to standard protocols (Upstate). Libraries for Illumina sequencing were made from two biological replicates per condition and were sequenced separately.

Bioinformatic analysis

Paired-end sequence reads were aligned to the mouse reference genome *mm10* using Bowtie2 (26) with parameters *-X 1000 –very-sensitive*, to map sequences up to 1 kb with maximum accuracy. We obtained ∼500 million pairs of

reads for both samples and a biological replicate experiment (Supplementary Table S1). Length distribution histograms for both biological replicate experiments are presented in Supplementary Figure S1B. The lengths of the paired-end reads were used to select the mono-nucleosomal DNA fragments with the lengths between 120 and 180 bp. The centers of the size-selected DNA fragments were used to define the positions of nucleosome dyads. The nucleosome occupancy profiles were obtained by stacking the size-selected reads. To account for copy number variation and biases resulting in uneven sequencing depth along the genome, the raw nucleosome occupancy at each bp x was normalized by the average occupancy in the window [x – 500, x + 500]. Single-end ChIP-seq data (GR, H2A.Z, Brg1) was first analyzed using the *SPP* package (27) (available at https://github.com/hms-dbmi/spp/) in order to estimate the average fragment length resulted in the sonication process. This average length was used to extend the fragments from the 5′ end that was sequenced toward the 3′ end: Brg1 reads were extended to a length of 100 bp, GR reads were extended to a length of 150 bp, and H2A.Z reads were extended to a length of 200 bp. The occupancy profiles were computed by stacking the extended reads. For the DNase-seq data, only the most 5′ nucleotides of the single-end reads were used to generate the profiles of DNase I cuts. To visualize specific loci, IGV browser (28) was used to load the tracks (tdf files) created with *igvtools*. GR binding sites were detected by MACS2 (29) using the following parameters *-g mm -B -q 1e-30*. GR enhancers were obtained from the list of GR binding sites by eliminating the sites from promoters (distance from TSS < 500 bp). RefSeq (30) annotations for the transcription start sites were obtained using the Table Browser tool from UCSC (https://genome.ucsc.edu/cgi-bin/hgTables). Heat maps representing different genomic data, aligned at GR enhancers were generated in MATLAB using the *Bioinformatics* toolbox to import data and the *heatmap* plotting function (http://www.mathworks.com/matlabcentral/fileexchange/24253-customizable-heat-maps). The average plots were also generated in MATLAB. The sequencing data from this study have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession numbers GSE92505 (MNase-seq data) and GSE94562 (ChIP-seq data).

RESULTS

Hormone-induced nucleosome shifts occur at responsive GR-enhancers

The majority of responsive GR-enhancers in mouse 3134 cells have a pre-programmed chromatin structure, as shown by their accessibility to DNase I prior to dexamethasone (Dex) treatment; i.e. the enhancer chromatin is already primed for GR binding, although ∼20% of responsive enhancers are weakly sensitive or inaccessible to DNase I (14). To examine the structural differences between pre-programmed and inaccessible enhancer chromatin, we mapped nucleosomes in untreated and Dex-treated 3134 cells genome-wide by MNase-seq. We selected the most highly occupied GR-enhancers (2746 sites) in 3134 cells for analysis using GR ChIP-seq data (14), aligned them on their GR peak summits, and sorted them to obtain a heat map with the most highly occupied sites at the top (Figure 1A). DNase I accessibility data (cut counts/bp) and nucleosome occupancy (MNase-seq) data were sorted in the same order (Figure 1B and C). Before Dex treatment, responsive GR-enhancers are generally accessible to DNase I and associated with a shallow NDR. After Dex treatment, the accessibility of these enhancers increased ∼2-fold on average (aggregate plot; Figure 1B) and correlated with GR occupancy (heat map; Figure 1A and B). The average nucleosome occupancy decreased after Dex, such that the NDR was deeper and wider, due to a net shift of nucleosomes away from the GR-enhancer (compare red and blue traces in Figure 1C). The heat map indicates that these Dex-induced nucleosome shifts are quite general (Figure 1C). Thus, at responsive GR-enhancers, hormone treatment induces GR binding and nucleosome shifts, such that the NDR expands and DNase I accessibility increases.

GR binds to both nucleosomal and nucleosome-depleted enhancers

Our observation that GR binds at mildly nucleosome-depleted enhancers is similar to that reported for GR binding in human ALL cells (17). However, we show below that this result is misleading. The aggregate plot (Figure 1C) shows a clear NDR, but it is quite shallow (the trough occurs at ∼80% of the genomic average occupancy), indicating that nucleosome occupancy is still quite high at occupied GR-enhancers. Given the ability of GR to bind a GRE-containing nucleosome with high affinity *in vitro* (11,31), we reasoned that the plot might represent the weighted average of two populations of GR-enhancers: nucleosomal and nucleosome-depleted.

To address this issue, we sorted the same set of GR-responsive enhancers according to the distance of the GR-peak from the nearest nucleosome occupancy peak in the absence of hormone, and aligned on the center of the nucleosome peak (Figure 2A, left heat map). The heat map shows nucleosome occupancy as a function of the distance between the GR binding site and the nearest nucleosome for all 2746 enhancers. The alignment produces a stripe of high nucleosome occupancy (red and yellow) down the middle of the heat map. The relationship between the nucleosome and the GR binding site can be understood by comparing this heat map with the GR occupancy heat map (Figure 2B, right panel). At the top of the nucleosome occupancy heat map (Figure 2A, left heat map), the GR-peak occurs within an NDR (blue) that is located just downstream of the major nucleosome peak, which is flanked by a second nucleosome peak farther downstream. The same is true at the bottom of the heat map, except that the NDR is located upstream of the major nucleosome peak. Nucleosome-depleted GR-enhancers are expected, given the analysis in Figure 1. However, the majority of GR-enhancers are not located in NDRs, but within a nucleosome (compare the central portion of the left heat map in Figure 2A with that of the right heat map in Figure 2B).

To illustrate this observation more precisely, we divided the 2746 GR-enhancers into ten groups according to their distance from the nucleosome dyad using 25 bp-increments

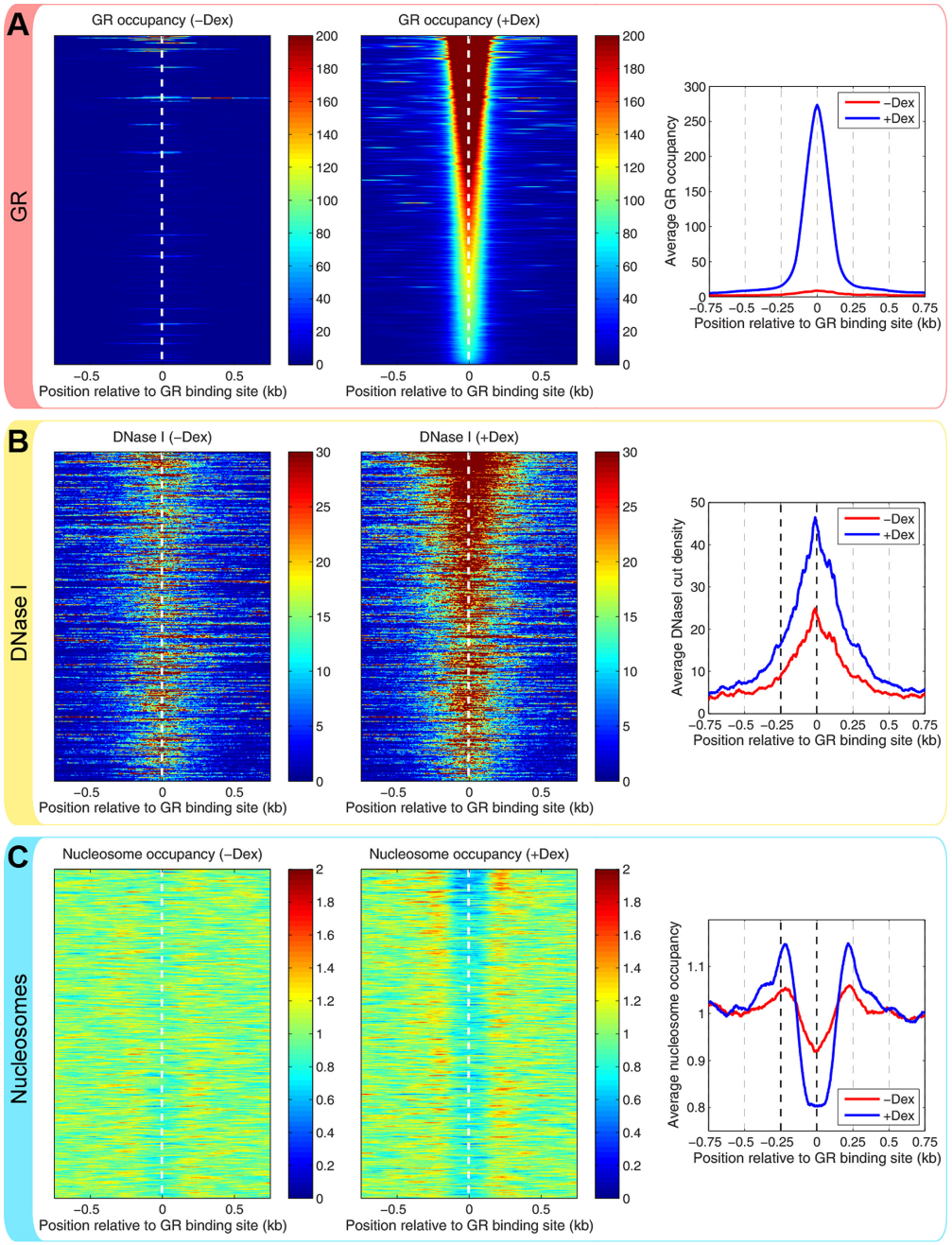

Figure 1. Hormone-induced nucleosome shifts occur at GR-responsive enhancers. Heat maps and aggregate plots for (**A**) GR occupancy, (**B**) DNase I accessibility (cut count density) and (**C**) nucleosome occupancy (MNase-seq) at strong GR-enhancers. Heat maps and aggregate plots. The 2746 most strongly bound GR-enhancers in 3134 cells identified by ChIP-seq (13) are aligned on the GR-peak observed with Dex and sorted by GR tag count (highest at the top). Before Dex treatment (left heat map); after Dex treatment (1 h) (right heat map). The GR peak is located at 0. The heat map scale is linear; red: high occupancy; blue: low occupancy.

(indicated by the white horizontal lines in the heat maps). The average nucleosome occupancy relative to the dyad of the central nucleosome was plotted for each group, together with the GR-peak to indicate the location of each group of GR-enhancers (Figure 2C). The top and bottom plots represent GR-enhancers located > 100 bp away from the nucleosome dyad; the GR peak is located within the NDR. There are similar numbers of GR binding events at enhancers located within 25 bp of the nucleosome dyad, at more peripheral enhancers (>25, 50 or 75 bp from the dyad), and at enhancers within NDRs (Figure 2C). Thus, the average amount of bound GR does not depend on the initial location of the enhancer relative to the nucleosome. Interestingly, GR-enhancers located within nucleosomes are not preferentially located near the nucleosome border, where increased accessibility is expected due to DNA end-breathing (32), but also well within the nucleosome, including at the dyad (central plots in Figure 2C). In conclusion, before

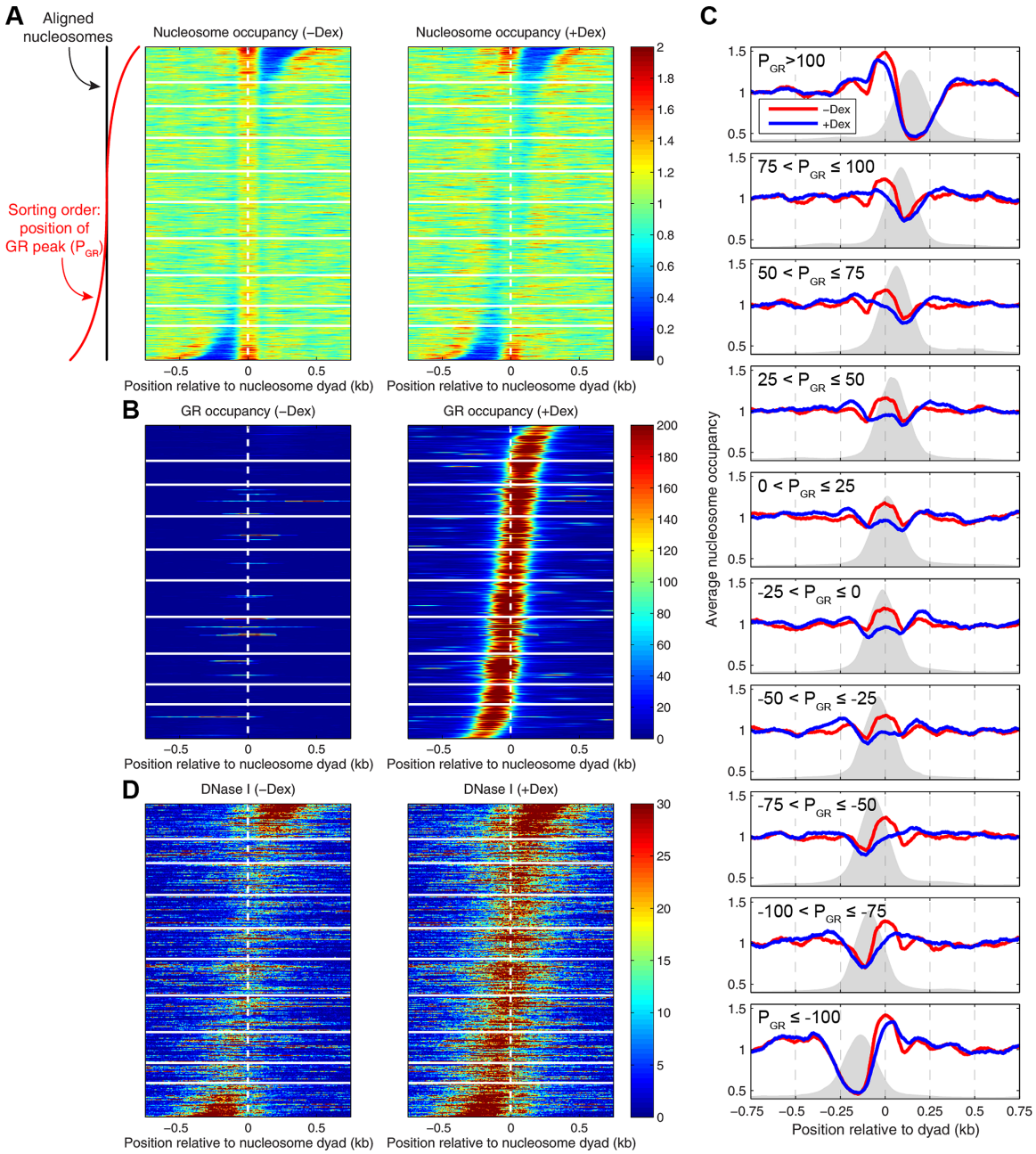

Figure 2. GR binds to both nucleosomal and nucleosome-depleted enhancers. (**A**) Nucleosome occupancy at GR-responsive enhancers. Heat maps for the same 2746 strongly-bound GR-enhancers described in Figure 1, re-aligned on the dyad of the nearest nucleosome before hormone treatment and sorted by the relative location of the GR-peak (red: high nucleosome occupancy; blue: low occupancy). The horizontal white lines separate these GR-enhancers into 10 groups depending on their distance from the nucleosomal dyad: >100 bp, between 100 and 75 bp, between 75 and 50 bp, between 50 and 25 bp and between 25 and 0 bp upstream or downstream of the nucleosomal dyad. (**B**) GR occupancy at nucleosome-depleted and nucleosomal GR-enhancers: ChIP-seq data for GR sorted and aligned as in A. (**C**) Aggregate plots for the 10 groups of GR-enhancers in A. The grey peak represents GR occupancy for each group. (**D**) DNase I accessibility of responsive enhancers.

hormone treatment, GR-enhancers adopt a continuum of states, ranging from almost nucleosome-free to high nucleosome occupancy. Furthermore, the location of the GR-enhancer relative to the nucleosome does not affect the level of GR binding after hormone treatment, indicating that GR can bind to nucleosomal sites.

After Dex treatment, nucleosome-depleted GR-enhancers show no change in nucleosome occupancy at the enhancer itself, but there is a small shift of the flanking nucleosome away from the GR-enhancer (Figure 2A, top and bottom of right heat map; Figure 2C, top and bottom plots). At nucleosomal GR-enhancers, Dex treatment results in reduced nucleosome occupancy over the enhancer, accompanied by subtle shifts in nucleosome occupancy away from the GR-enhancer (Figure 2A, central portion of right heat map; Figure 2C, central plots). This

observation suggests that the nucleosome covering the GR-enhancer may be removed or shifted aside in response to hormone-induced GR binding.

Motif analysis using MEME identified the expected GR binding site motif when all 2746 enhancers are considered together (approximating to GnACAnnnTGTnC). However, analysis of the motif for each group of enhancers revealed that the motif at nucleosome-depleted enhancers (groups 1 and 10) lacks one of the two outer bases (GnACAnnnTGT; note the symmetry of the site), suggesting that slightly weaker GREs may be bound at nucleosome-depleted enhancers (Supplementary Figure S2).

Nucleosomal GR-responsive enhancers can be hypersensitive or insensitive to DNase I

In the absence of hormone, nucleosome-depleted GR-enhancers are more accessible to DNase I than nucleosomal GR-enhancers (Figure 2D, heat map at left), suggesting that nucleosomes reduce accessibility to DNase I. However, the DNase I signal over nucleosomal GR enhancers is somewhat patchy (Figure 2D; groups 2–8 in heat map at left), suggesting that some nucleosomal enhancers are hypersensitive to DNase I and some are not. We investigated this issue by re-sorting the nucleosomal enhancers (defined as those with a nucleosomal occupancy at least 0.75 times the genomic average) by DNase I hypersensitivity and dividing them into quintiles (Figure 3A). Prior to hormone treatment, the DNase I hypersensitivity of nucleosomal GR-responsive enhancers ranged widely from ∼20 times the genomic average to virtually background (Figure 3A), even though nucleosome occupancy over the GR site is very similar for all quintiles (Figure 3B). The corresponding heat maps are presented in Supplementary Figure S3. There appear to be two types of nucleosomal GR-responsive enhancer: DNase I-accessible (the top two quintiles in Figure 3A) and DNase I-insensitive (bottom two quintiles). Although GR binding is somewhat higher at nucleosomal enhancers in the top quintile than in the bottom quintile (Figure 3C), GR binds strongly to DNase I-insensitive enhancers, indicating that the structural alteration in the nucleosome presumably responsible for DNase I hypersensitivity is not essential for GR to bind to a nucleosomal site.

After hormone treatment, nucleosome-depleted enhancers and all nucleosomal GR-responsive enhancers except for the lowest quintile (Figure 3A), become more accessible to DNase I (compare aggregate plots and heat maps in Figures 2D, 3A and Supplementary Figure S3). The increase in DNase I accessibility correlates quite well with decreased nucleosome occupancy after hormone treatment (Figure 3A and B; Supplementary Figure S3). It is important to note that these DNase I-sensitive nucleosomes are canonical as defined by MNase digestion; they protect ∼150 bp of DNA after extensive MNase digestion and are therefore not MNase-sensitive 'fragile' nucleosomes (see (33)).

Our observation that some nucleosomal GR-enhancers are accessible to DNase I indicates that hypersensitivity does not simply reflect the presence of an NDR. Consistent with this, there is a modest anti-correlation between the nucleosome occupancy in a 250-bp window centered on the GR peak and DNase I accessibility (Pearson $R =$ –0.30; Supplementary Figure S4). The correlation between GR binding and DNase I accessibility is better ($R = 0.48$; Supplementary Figure S4). Most importantly, there is no anti-correlation between GR binding and nucleosome occupancy ($R = -0.05$), suggesting that GR binds nucleosomal and nucleosome-depleted enhancers with similar affinities.

H2A.Z marks nucleosome-depleted enhancers and DNase I-accessible nucleosomal enhancers but not DNase I-inaccessible nucleosomal enhancers

Active enhancers are often marked by histone exchange or turnover, particularly of H2A.Z (34,35). We measured H2A.Z incorporation into nucleosomes at GR-enhancers in 3134 daughter cell lines using a Tet-off system expressing Flag-tagged H2A.Z under the control of the tetracycline repressor. To minimize incorporation of tagged H2A.Z into nucleosomes assembled during replication, H2A.Z expression was limited to 24 hours after tetracycline removal. As expected, in the presence of tetracycline, Flag-H2A.Z is assembled into GR-enhancer chromatin at a very low level because its expression is repressed (Figure 4A). After tetracycline removal to induce H2A.Z expression, the nucleosomes flanking nucleosome-depleted GR-enhancers are marked by H2A.Z (data sorted as in Figure 2; top and bottom of the heat map; Figure 4B and C), as are some nucleosomal GR-enhancers (central portion of heat maps in Figure 4B and C). The H2A.Z peaks are not as well resolved as nucleosomes in the nucleosome occupancy maps, because the resolution of ChIP-seq is lower than that of MNase-seq. Hormone treatment increases H2A.Z incorporation into nucleosomes at both types of GR-enhancer (Figure 4C).

For a more quantitative representation, we determined the average H2A.Z incorporation as a function of the distance of the GR-enhancer from the central nucleosome for the same ten groups of enhancers described in Figure 2 (Figure 4D). At nucleosome-depleted enhancers (top and bottom plots in Figure 4D), the flanking nucleosomes incorporate H2A.Z and shift farther apart in response to hormone, whereas at nucleosomal enhancers, there is a single broad peak of H2A.Z prior to hormone treatment, which resolves into two overlapping peaks after hormone treatment (central plots in Figure 4D). Thus, H2A.Z distribution follows the hormone-dependent nucleosome shifts observed at both types of GR-enhancer.

H2A.Z incorporation at DNase I hypersensitive and insensitive nucleosomal GR-enhancers was examined by sorting them according to DNase I accessibility (Figure 3D; Supplementary Figure S3). In the absence of hormone, only the more hypersensitive nucleosomal GR-enhancers were strongly marked by H2A.Z, represented by a broad ChIP peak centered on the GR-binding site (Figure 3D). After hormone treatment, a larger fraction of nucleosomal GR-enhancers incorporated H2A.Z (Figure 3D), correlating with increased DNase I hypersensitivity (Figure 3A). However, the DNase I-insensitive nucleosomal enhancers (bottom quintiles in Figure 3D) incorporated very little H2A.Z even though GR was bound (Figure 3C).

In conclusion, DNase I-hypersensitive GR-responsive enhancers are marked by relatively high levels of H2A.Z incorporation into the nucleosomes covering the enhancer

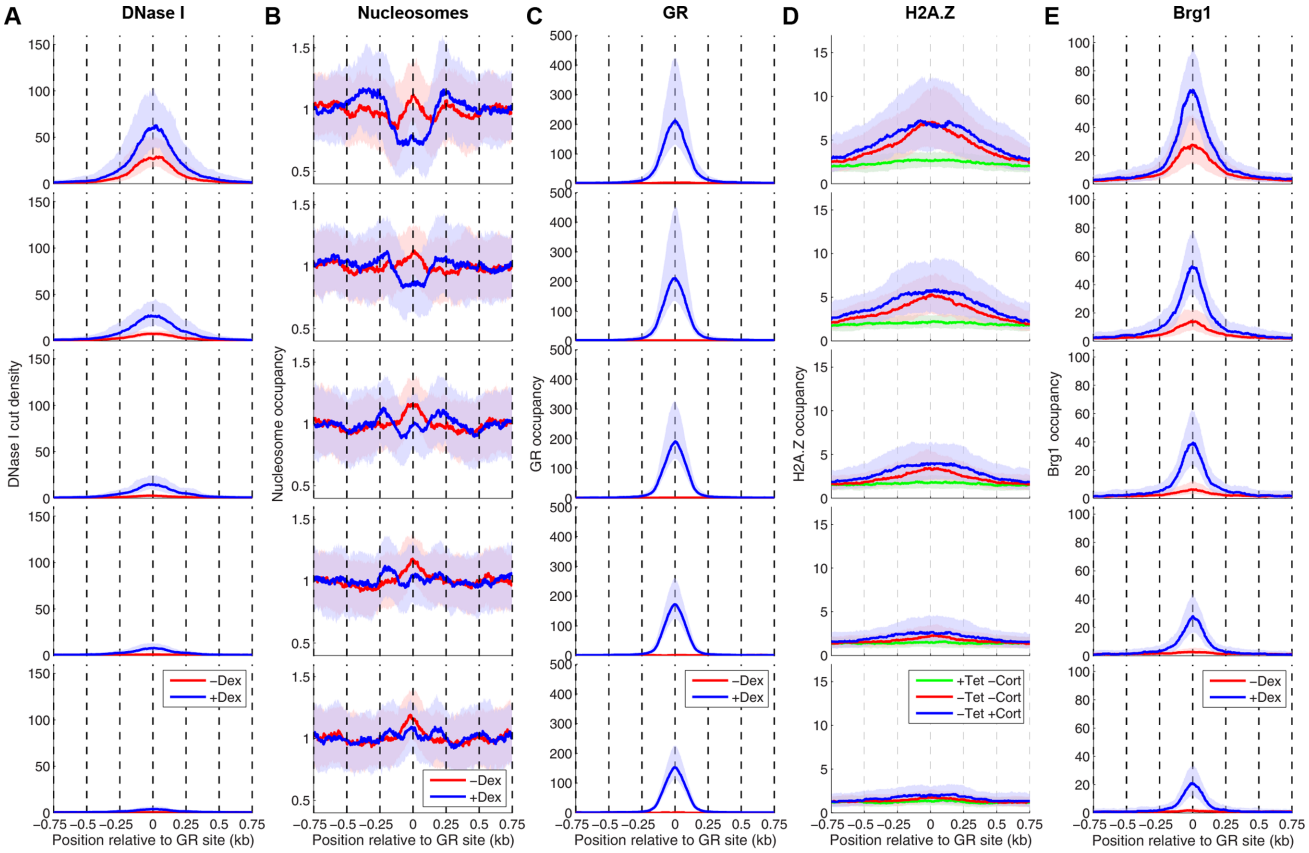

Figure 3. Nucleosomal GR-responsive enhancers can be hypersensitive or insensitive to DNase I. Nucleosomal GR-enhancers (those with a nucleosome occupancy >0.75 times the genomic average) were sorted by DNase I hypersensitivity and divided into quintiles, such that quintile 1 contains the most hypersensitive nucleosomes and quintile 5 contains the least sensitive nucleosomes. Aggregate plots for each quintile (red: no Dex; blue: + Dex), showing the medians (solid lines) and 25–75 percentile ranges of the data (filled areas). (**A**) DNase I hypersensitivity. (**B**) Nucleosome occupancy. (**C**) GR occupancy. (**D**) H2A.Z incorporation (see Figure 4). Green line: +Tet control. (**E**) Brg1 occupancy (see Figure 5). See Supplementary Figure S3 for the corresponding heat maps.

or immediately adjacent to it, whereas DNase I-insensitive nucleosomal enhancers lack H2A.Z. This observation suggests that H2A.Z incorporation may contribute to DNase I sensitivity. Indeed, DNase I cut density correlates better with H2A.Z incorporation than with nucleosome occupancy (R = 0.50 and –0.30, respectively; Supplementary Figure S4). This suggests that DNase I accessibility may reflect both the presence of weakly protected DNA at nucleosome-depleted enhancers and the transient exposure of nucleosomal DNA during H2A.Z exchange at some nucleosomal enhancers. GR binding correlates quite well with H2A.Z incorporation (R = 0.25; Supplementary Figure S4), suggesting that H2A.Z exchange may facilitate GR binding, even though it is not necessary for binding.

Brg1 is associated with nucleosome shifts at all GR-responsive enhancers

The nucleosome shifts that occur at GR-responsive enhancers presumably reflect the activities of ATP-dependent chromatin remodeling enzymes, such as Brg1, Snf2H and Chd4, which are associated with accessible chromatin (14,22). We addressed the role of Brg1 in setting the chromatin structure of nucleosome-depleted and nucleosomal GR-enhancers using ChIP-seq data for Brg1 in 3134 cells (22) (Figure 5A). Before Dex, Brg1 is strongly associated with nucleosome-depleted enhancers (Figure 5A, top and bottom of the heat map). In contrast, Brg1 levels are much lower at most nucleosomal enhancers (center of heat map in Figure 5A). The nucleosomal enhancers with high levels of Brg1 correspond to the most DNase I-sensitive enhancers (Figure 3E; Supplementary Figure S3). Thus, DNase I-hypersensitive nucleosomal enhancers are associated with Brg1 prior to hormone treatment, but DNase I-insensitive nucleosomal enhancers lack Brg1.

After Dex treatment, all types of GR-responsive enhancer are strongly associated with Brg1 (Figure 5B, C), including DNase I-insensitive nucleosomal enhancers (Figure 3E). MACS analysis of Brg1 peaks present before and after hormone treatment indicated that very few (only 10) Brg1 peaks disappeared in response to hormone, suggesting that a major Brg1 redistribution did not occur, although small decreases in Brg1 levels at multiple sites could perhaps account for the increased Brg1 levels at GR-enhancers after hormone treatment. Alternatively, the hormone-induced Brg1 peaks may derive from a pool of non-chromatin-bound Brg1. Thus, Brg1 is already present at nucleosome-depleted GR-enhancers, where the nucleosomes have already been shifted or removed to create an NDR, whereas it

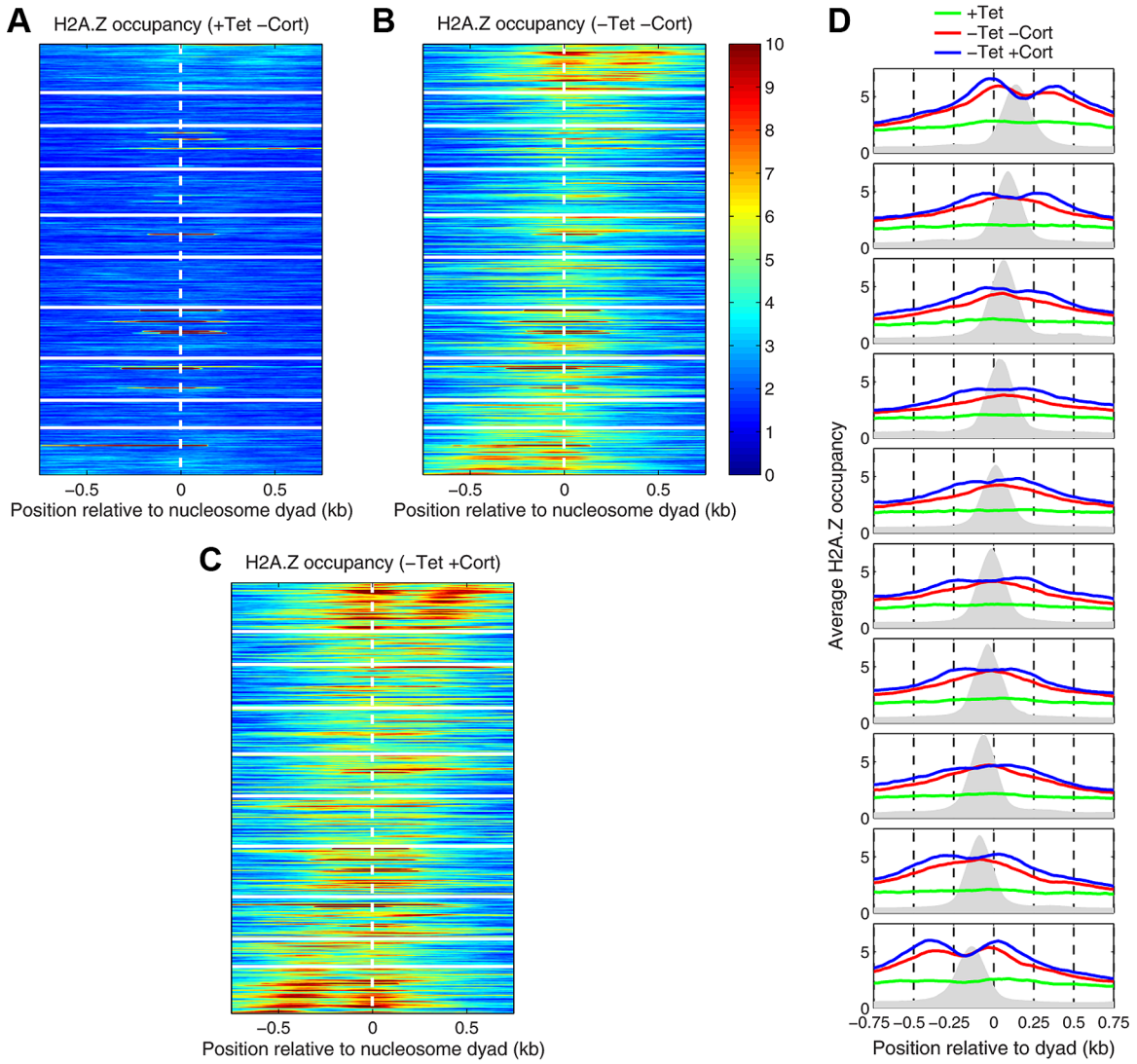

Figure 4. H2A.Z marks nucleosome-depleted and DNase I-accessible nucleosomal GR-enhancers. Flag-tagged H2A.Z was expressed using a Tet-Off system and detected by ChIP-seq. Heat maps showing H2A.Z occupancy with GR-enhancers sorted as in Figure 2A. (**A**) Before tetracycline (Tet) removal. (**B**) After Tet removal (24 h). (**C**) After Tet removal and 1 h corticosterone (Cort) treatment. (**D**) Aggregate plots for the 10 groups of GR-enhancers in Figure 2A. Grey peak: GR occupancy.

is apparently recruited to DNase I-insensitive nucleosomal enhancers in response to hormone, coincident with a shift in the nucleosome, for which it may be responsible. Indeed, Brg1 binding correlates well with DNase I accessibility, GR-binding, H2A.Z exchange and nucleosome occupancy (R = 0.72, 0.53, 0.51 and –0.29, respectively; Supplementary Figure S4).

GR enhancers specific to another cell type are nucleosomal and incorporate very little H2A.Z

Previously, we identified a set of ∼750 GR-enhancers to which GR binds in mouse pituitary AtT-20 cells but not in 3134 cells (14). In 3134 cells, these AtT-20-specific GR-enhancers are unresponsive to hormone: they do not bind GR (Figure 6A) and they are inaccessible to DNase I both before and after Dex treatment (Figure 6B). In untreated 3134 cells, AtT-20-specific GR-enhancers are almost all nucleosomal, with a small peak in nucleosome occupancy (Figure 6C), as observed at nucleosomal GR-responsive enhancers. However, after hormone treatment the nucleosomal peak persists (Figure 6C), unlike at responsive enhancers (compare with Figure 2C). Unresponsive GR-enhancers have only slightly higher levels of H2A.Z than the flanking nucleosomes (Figure 6D), which correlate with the slightly higher nucleosome occupancy (Figure 6C), and are unaffected by hormone (Figure 6D). They also have much lower levels of Brg1 (Figure 6E). In conclusion, unresponsive (AtT-20-specific) GR-enhancers are inaccessible to DNase I, generally nucleosomal, and have low levels of Brg1 and H2A.Z.

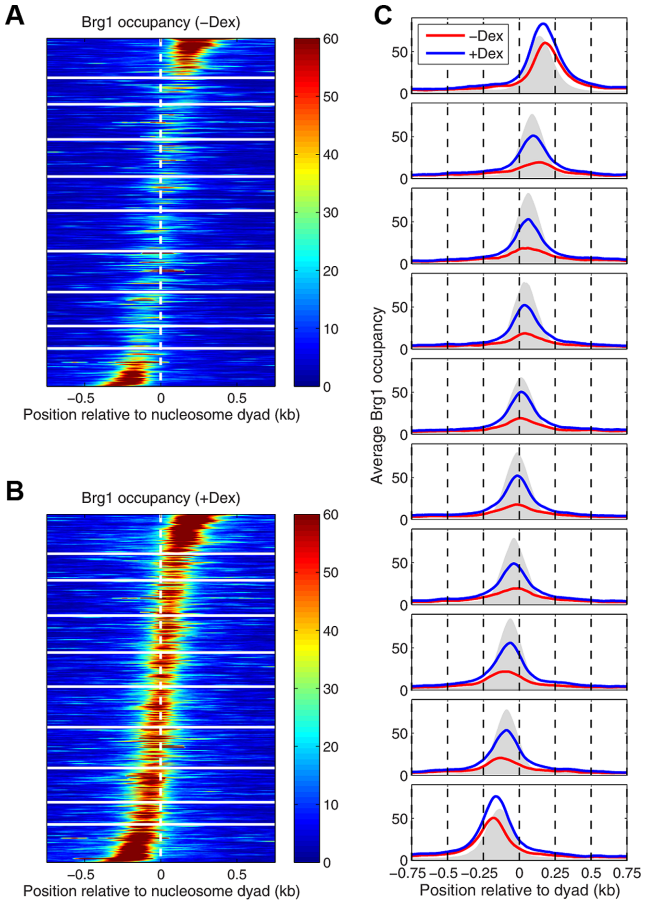

Figure 5. Brg1 is associated with nucleosome shifts at all GR-responsive enhancers. Heat maps showing Brg1 occupancy (ChIP-seq data) with GR-enhancers sorted as in Figure 2A. (**A**) No Dex. (**B**) After 1 h Dex treatment. (**C**) Aggregate plots for the 10 groups of GR-enhancers defined in Figure 2A. Gray peak: GR occupancy.

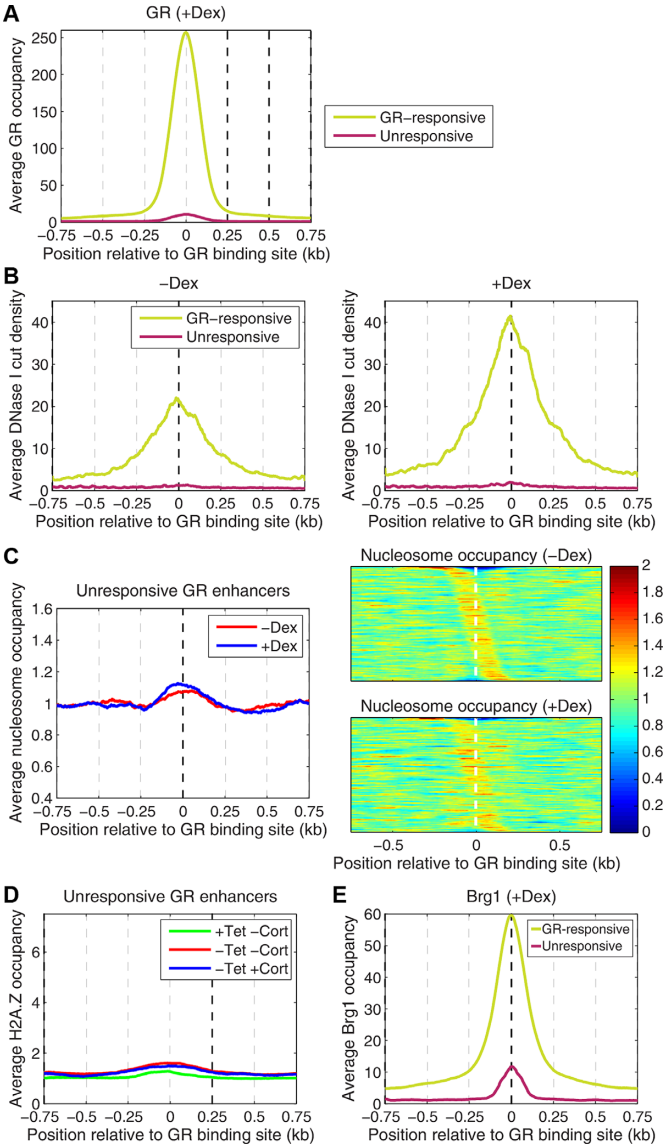

Figure 6. GR-unresponsive enhancers are nucleosomal and incorporate very little H2A.Z. Comparison of responsive and unresponsive GR-enhancers in 3134 cells (the latter are bound by GR in AtT-20 pituitary cells but not in 3134 cells (14)). All data shown here are for 3134 cells. (**A**) GR occupancy after Dex treatment. (**B**) DNase I accessibility before and after Dex treatment (1 h). (**C**) Nucleosome occupancy at unresponsive GR-enhancers before and after Dex treatment (aggregate plot). Heat maps show unresponsive GR-enhancers only, sorted according to the distance of the nearest nucleosome to the GR peak in the absence of hormone. (**D**) H2A.Z incorporation. (**E**) Brg1 occupancy after Dex.

DISCUSSION

Nucleosome-depleted and nucleosomal GR-enhancers

We have shown that responsive GR-enhancers in mouse 3134 cells can be located within a pre-existing NDR (nucleosome-depleted) or within a nucleosome (Figure 7). Nucleosome-depleted GR-enhancers are already associated with Brg1 and flanked by H2A.Z-incorporating nucleosomes (Figure 7B). Nucleosomal GR-enhancers may be divided into two classes: (i) DNase I-hypersensitive, H2A.Z-containing nucleosomes associated with Brg1 (Figure 7C); (ii) DNase I-insensitive nucleosomes lacking H2A.Z and Brg1 (Figure 7D). In the presence of hormone, GR binds to both types of nucleosomal enhancer and to nucleosome-depleted enhancers at similar average occupancies and more Brg1 is recruited, presumably accounting for the nucleosome shifts away from the GRE. In contrast, GR-enhancers that are active in another cell type (AtT-20) are nucleosomal, inaccessible and resistant to H2A.Z incorporation (Figure 7A). Specific examples of these chromatin states are shown in Supplementary Figure S5.

We propose that the pre-existing NDRs at nucleosome-depleted GR-enhancers are created by other transcription factors bound in the absence of hormone which, with the assistance of Brg1, shift the H2A.Z-nucleosomes aside (Figure 7B). The GRE is relatively exposed, facilitating the loading of GR after hormone treatment (36). On the other hand, at responsive nucleosomal enhancers, GR binds to a nucleosome and, together with Brg1, may assist with the loading of downstream factors by initiating the formation of an NDR (36). On average, GR occupies both types of enhancer to similar extents, suggesting that the nucleosome does not strongly inhibit GR binding, as expected from the facile binding of GR to a nucleosomal GRE *in vitro* (11),

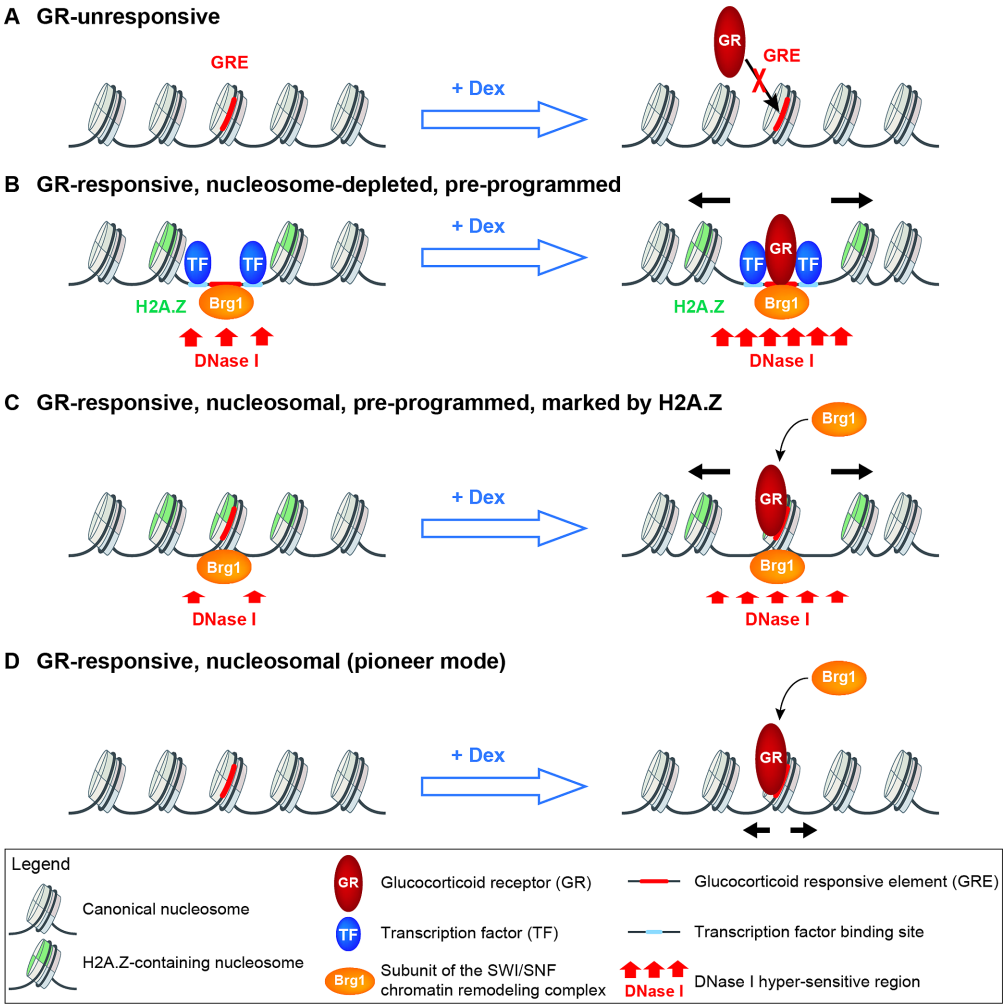

Figure 7. Conventional and pioneer modes of GR binding to responsive enhancers. (A) GR does not bind to unresponsive enhancers. They are nucleosomal, inaccessible to DNase I, do not incorporate H2A.Z and lack Brg1. (**B**) GR binds to pre-programmed nucleosome-depleted enhancers. The NDR may be created by other transcription factors bound to their cognate sites near the GRE, in concert with the Brg1 complex, a chromatin remodeler capable of shifting nucleosomes. The NDR is flanked by nucleosomes incorporating H2A.Z and is accessible to DNase I. In response to hormone, GR binds to the GRE in the NDR and the flanking nucleosomes are shifted aside (black arrows), presumably by Brg1. (**C**) GR binds to pre-programmed DNase I-accessible nucleosomal enhancers. The GRE is covered by a nucleosome incorporating H2A.Z and associated with Brg1, both contributing to DNase I accessibility. In response to hormone, GR binds to the GRE, recruits more Brg1 and the nucleosome is shifted aside. (**D**) GR binds to DNase I-insensitive nucleosomal enhancers. The GRE is covered by a nucleosome which lacks H2A.Z and Brg1. In response to hormone, GR binds to the GRE, recruits Brg1, and the nucleosome is shifted.

although the affinity of GR for a nucleosomal GRE is 2- to 10-fold lower than for a protein-free GRE, depending on its location within the nucleosome (12,13). Our data indicate that GR behaves like a pioneer factor, because it binds to GREs located within nucleosomes. Although H2A.Z incorporation may facilitate binding, it is not essential for GR binding *in vivo*. H2A.Z exchange can account for our observation that responsive nucleosomal enhancers and unresponsive enhancers have very similar nucleosome occupancies, but very different accessibilities to DNase I. We propose that DNase I accessibility reflects a combination of nucleosome depletion, nucleosome shifts and H2A.Z exchange. That is, DNase I detects dynamic chromatin.

Facile binding of GR to a nucleosomal GRE does not explain why GR does not bind to unresponsive GR-enhancers, which are also nucleosomal (Figure 7A and D). The sensitivity of GR binding to DNA methylation provides a potential explanation, since unresponsive enhancers may be more methylated than responsive enhancers (3). In addition, unresponsive enhancer nucleosomes may be associated with heterochromatin-like features that prevent GR binding.

At all types of responsive enhancer, the net effect of hormone-induced GR binding is a nucleosome shift that would increase exposure of the GR binding site. These nucleosome shifts correlate with the presence of the SWI/SNF-related Brg1 complex, an ATP-dependent chromatin remodeler capable of moving and displacing nucleosomes. Brg1 is required for hormone receptor-mediated gene activation and interacts directly with GR (37–40), accounting for its recruitment to nucleosomal GR-enhancers after hormone treatment and coincident nucleosome shifts.

On the other hand, other factors presumably recruit Brg1 to nucleosome-depleted GR-enhancers because Brg1 is bound prior to hormone treatment, although more Brg1 is recruited in response to hormone. Apparently, the nucleosomes have already been shifted to create the NDR at these GR-enhancers.

MNase-seq experiments performed on human ALL cells suggest that GR-enhancers are associated with an NDR, with little change in response to Dex (17). This differs from our observations in mouse cells, where we also detect a modest NDR at the average GR-enhancer prior to hormone, but the NDR deepens as nucleosomes are shifted aside in response to hormone (Figure 1A). These observations may be reconciled by considering the strength of GR binding, since we limited our analysis to strongly bound GR-enhancers (the top 2746 of the 8236 originally reported (14)); inclusion of weakly bound GR-enhancers would reduce the magnitude of the effects on the NDR. Wu *et al*. (17) found that subsets of GR-enhancers are marked with di-methylated H3-K4 and form a deeper NDR in response to hormone, although the observed shifts in H3-K4 di-methylated nucleosomes may not be representative of all nucleosomes (i.e. H3-K4 di-methylated nucleosomes may behave differently from unmodified nucleosomes). More importantly, we have shown that many GR-enhancers are nucleosomal (Figure 2), which are undetected by our analysis in Figure 1 or by Wu *et al*. (17). Histone modifications such as H3–K4 mono-methylation and H3-K27 acetylation mark a large fraction of GR-enhancers and may be important for GR binding (17). More generally, poised enhancers are marked by H3–K27 methylation, whereas transcriptionally active enhancers producing eRNAs are marked by a transition to H3–K27 acetylation (7,16,41). However, how these chromatin marks relate specifically to GR-enhancers remains to be established.

Chromatin structure of other steroid receptor enhancers

Our analysis of GR binding at enhancers clarifies the comparison with other hormone receptors. We find that a significant minority of GR-responsive enhancers are nucleosome-depleted. However, AR, PR and ER enhancers are predominantly nucleosomal (18–21), suggesting that these hormone receptors do not often bind at pre-existing NDRs, unlike GR. Hormone-induced AR binding results in nucleosome eviction, although this conclusion is based on the distribution of di-methylated H3-K4 nucleosomes (18), which may not be typical of all nucleosomes. PR binds to a PRE assembled into a very high occupancy nucleosome which is remodeled after progesterone treatment, generating a sub-nucleosome (20). However, even after hormone treatment, the occupancy of the PRE-nucleosome remains much higher than the flanking chromatin implying, presumably incorrectly, that the rest of the genome is strongly depleted of nucleosomes. A possible explanation is that the PR-samples were under-digested by MNase, under which conditions PRE-nucleosomes might be preferentially released and therefore over-represented. Our results suggest that GR binds nucleosomal enhancers, resulting in some nucleosome re-positioning to create a weak NDR. In contrast to PR (20) and ER (21), there is little evidence for GR-mediated remodeling to sub-nucleosomes, as this would result in a loss of canonical nucleosomes, which we do not observe, if the nucleosome shifts are taken into account. Thus, GR may well behave differently from the other hormone receptors, in that it binds GR-enhancer nucleosomes, resulting in some re-positioning but little remodeling of their structure. However, the fact that some GR-enhancer nucleosomes incorporate H2A.Z indicates that they must at least transiently pass through a sub-nucleosomal state. These observations can be reconciled by arguing that, in the case of GR, the remodeled nucleosome associated with H2A.Z exchange is short-lived and therefore undetected in our MNase-seq data.

ACCESSION NUMBERS

GEO database entries: GSE92505 (MNase-seq data); GSE94562 (H2A.Z and Brg1 ChIP-seq data).

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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