Nucleosome-Driven Transcription Factor Binding and Gene Regulation

Cecilia Ballaré,1,2 Giancarlo Castellano,1,2 Laura Gaveglia,1,2 Sonja Althammer,2 Juan González-Vallinas,2 Eduardo Eyras,2,3 Francois Le Dily,1,2 Roser Zaurin,1,2 Daniel Soronellas,1,2 Guillermo P. Vicent,1,2 and Miguel Beato1,2,*

1 Gene Regulation Stem Cells and Cancer Program, Centre for Genomic Regulation (CRG), 08003 Barcelona, Spain
2 University Pompeu Fabra (UPF), 08002 Barcelona, Spain
3 Institució Catalana de Recerca i Estudis Avançats, Catalonia (ICREA), 08010 Barcelona, Spain
*Correspondence: miguel.beato@crg.es
http://dx.doi.org/10.1016/j.molcel.2012.10.019

SUMMARY

Elucidating the global function of a transcription factor implies the identification of its target genes and genomic binding sites. The role of chromatin in this context is unclear, but the dominant view is that factors bind preferentially to nucleosome-depleted regions identified as DNase-hypersensitive sites (DHS). Here we show by ChIP, MNase, and DNase assays followed by deep sequencing that the progesterone receptor (PR) requires nucleosomes for optimal binding and function. In breast cancer cells treated with progesterins, we identified 25,000 PR binding sites (PRbs). The majority of these sites encompassed several copies of the hexanucleotide TGGTYCY, which is highly abundant in the genome. We found that functional PRbs accumulate around progesterone-induced genes, mainly in enhancers. Most of these sites overlap with DHS but exhibit high nucleosome occupancy. Progestin stimulation results in remodeling of these nucleosomes with displacement of histones H1 and H2A/H2B dimers. Our results strongly suggest that nucleosomes are crucial for PR binding and hormonal gene regulation.

INTRODUCTION

Gene expression in a differentiated eukaryotic cell type is determined by its specific set of transcription factors (TFs) and by the particular organization of its genome in chromatin. Understanding how sequence-specific TFs control gene regulatory networks requires knowledge of their intracellular interactions and, in particular, of their genomic binding sites, which are influenced by the cell-specific chromatin structure (Ram et al., 2011). But how the nucleosomal organization and the higher order chromatin structure of these genomic regions influence access of TFs to their target DNA sequences is still a matter of debate.

Steroid hormones exert their effects by binding to intracellular receptors, which regulate gene expression mainly by interacting with specific DNA sequences in chromatin and recruiting chromatin remodeling complexes and transcriptional coregulators (Beato et al., 1995). In addition, hormone receptors can signal via crosstalk with kinase signaling cascades (Migliaccio et al., 1999). In breast cancer cells, a small fraction of the estrogen and progesterone receptors (ER and PR, respectively) is attached to the cytoplasmic side of the cell membrane via a palmitoylated residue (Ballaré et al., 2003; Pedram et al., 2007). When activated upon hormone binding, these membrane receptors interact with members of the c-SRC family of protein tyrosine kinases, leading to activation of various downstream kinase cascades, including ERK1/ERK2 (Migliaccio et al., 1996, 1999).

We have previously shown that hormone-activated ERK1/ERK2 phosphorylates PR and the downstream kinase MSK1 in the cell nucleus. The resulting ternary complex pPR/pERK/ pMSK1 is the active form of the receptor that interacts productively with chromatin targets, where pMSK1 phosphorylates histone H3 at S10 (Vicent et al., 2006). This modification contributes to displacement of a repressive complex containing HP1γ (Vicent et al., 2006). Recently, we found that already 1 min after hormone treatment, pPR interacts with and activates the CyclinA/CDK2 complex, which is recruited to regulated promoters and phosphorylates histone H1, leading to its NURF-mediated displacement. This process requires trimethylation of histone H3 at lysine 4 by MLL2/3 of the ASCOM complex that stabilizes the NURF complex (Vicent et al., 2011). Subsequently, the pPR ternary complex recruits the BAF complex that catalyzes displacement of histones H2A/H2B dimers. Local displacement of histones H1 and H2A/H2B is needed for optimal induction of progestin-target promoters. Thus, crosstalk of PR with kinase signaling pathways impinges on the promoter chromatin structure as requisite for gene regulation (Beato and Vicent, 2011).

Although there is evidence for receptor binding to hormone responsive elements (HREs) organized in nucleosomes in the MMTV promoter, we also find HREs that are not accessible within nucleosomes (Truss et al., 1995). How the chromatin organization of the hormone responsive regions influences access of hormone receptors to the HREs genome-wide is still not clear. The generally accepted view is that hormone receptors prefer binding to HREs located in nucleosome-depleted “open” chromatin regions that are marked by so-called pioneer factors, such as FoxA1 and AP1 (Hurtado et al., 2011; Carroll et al., 2005; John et al., 2011; Biddie et al., 2011). To approach this issue for PR, we have identified the PR binding sites genome-wide and...
performed global gene expression analysis, DNaseI sensitivity assays, and nucleosome mapping experiments in breast cancer cells. Intriguingly, we find that PR binds only a tiny fraction of potential HREs, which are partly marked by a higher sensitivity to DNaseI but enriched in nucleosomes prior to hormone addition. These nucleosomes encompassing PR binding sites are remodeled but not evicted upon hormone induction. Our results strongly suggest that the nucleosomal organization of HREs favors optimal PR binding and hormonal induction.

RESULTS

Identification of PR Binding Sites and Their Relationship to Gene Regulation

Using chromatin immunoprecipitation with an antibody against PR, followed by deep sequencing (ChIP-Seq), we found 25,876 PR binding sites (PRbs) in the human breast cancer cells T47D-MTVL (Truss et al., 1995) treated for 30 min with the synthetic progestin R5020 (10 nM). Most of these sites were also found after 5, 60, or 360 min hormone treatment (Figure 1A and Figure S1A available online). Examples and validation of the results are shown in Figure 1B and S1B, respectively. The large majority of the PRbs were located in introns and intergenic regions (Figure S1C). However, PRbs density was highest around the transcription start sites (TSS), and PRbs were enriched in genic versus intergenic regions (Figure S1D).

To correlate PRbs with gene regulation, we performed whole-genome expression microarrays in cells stimulated 0, 1, and 6 hr with R5020. We found 3,835 genes differentially expressed following R5020 treatment, of which 1,989 were upregulated (Figure 1C), including a significant number of genes involved in cell-cycle progression, proliferation, control of apoptosis, cell signaling, and general metabolism (Figures S2A and S2B), concordant with the initial proliferative burst induced by progesterone in T47D cells (Groshong et al., 1997). Among the repressed genes, we found signaling molecules associated with cell-growth arrest and control of apoptosis, antiproliferative cytokines and their receptors, and multiple transcriptional repressors and corepressors (Figures S2A and S2B).

Comparison of gene expression results with PR occupancy in response to hormone treatment showed a significant

Nucleosomes Facilitate Genome-wide PR Binding
enrichment of PRbs around R5020-regulated genes, especially for the upregulated ones (Figure 1D). In fact 68% of the induced and 35% of the repressed genes contained at least one PRbs in the proximity (in a window from −10 kb upstream of the TSS to +5 kb downstream of the transcription termination site [TTS]), and these values increased to 89% and 68%, respectively, by extending the window up to −100 kb from TSS to +25 kb from TTS. Among the R5020-induced genes, a positive correlation was found between the magnitude of hormone response and the amount of associated PRbs (Figure 1E). This correlation suggests an additive effect of multiple PRbs in the induction of target genes.

**Redefinition of the Consensus Progesterone Responsive Element**

We have screened the identified PRbs for the consensus sequences corresponding to the previously reported specific progesterone responsive element (PRE) motif and for the more general GREF matrix family—a group of matrices for PR, glucocorticoid, and androgen receptor. Only 46% of the PRbs contained at least one match for the PRE matrix and 79% for the less specific GREF matrix family (Figure 1F). To refine the PRE matrix, we analyzed the sequence of the 10% most significant PRbs and defined a consensus motif (Figure 1G), which was found in up to 89% of all PRbs, with an average of 2.5 matches per site (Figure 1F). Therefore, the PRE here identified is a better predictor of PRbs than the previously used PRE or GREF consensus.

Nevertheless, the examination of the complete human genome reveals 8.17 million of such PRE matches. Thus, PR binds only 0.3% of potential PREs, indicating that the presence of the PRE sequence is not sufficient to recruit PR. A detailed sequence analysis of the PRbs showed that most of them consist of clusters of several PRE sequences (Figure S3A). However, these clusters are still very frequent in the genome, and therefore additional factors must account for the very small proportion of them that are actually bound.

One possible reason could be the need for cooperation with other TFs. PRbs showed an enrichment of binding sites for members of several TF families, including Ets, STAT, FKHD, AP1, and SP1 (Table S1 and Figure S3B), but none of them was significant enough to explain the large discrepancy between potential PRbs and actual bound sites.

**Functional PRbs Are Marked by High Nucleosome Occupancy and Undergo Nucleosome Remodeling after Hormone Stimulation**

Previous reports have shown that glucocorticoid receptors bind preferentially to sites within regions of “open” chromatin, detected as DNase1 hypersensitive sites (DHS) (John et al., 2008) and usually represented as nucleosome depleted. However, no measurement of nucleosome occupancy over DHS has been reported. We therefore used DNase1-sequencing (DNase1-seq) to determine the genome-wide location of DHS (Boyle et al., 2008) in T47D-MTVL cells before and after hormone treatment. Genomic regions that bind PR in the presence of progestin exhibited a high sensitivity to DNase1 before hormone stimulation, which increased after progestin treatment (Figures 2A and S4A).

To test whether these sites are indeed depleted of nucleosomes, we performed genome-wide nucleosome mapping using MNase digestion and deep sequencing in cells untreated or stimulated with R5020. Unexpectedly, before hormone treatment we found high nucleosome occupancy over the regions where PR will bind after stimulation (Figure 2B, red line). The width of the peak was around 350 nt, suggesting a localized process affecting one or two nucleosomes. This nucleosome enrichment was significantly higher compared to a set of random genomic sequences of the same length or to sequences containing potential PREs that were not bound by PR in T47D-MTVL cells (Figures S4B and S4C). Moreover, PRbs lacking the consensus PRE sequence showed the same high nucleosome enrichment as the ones carrying PRE motifs (data not shown). Thus, in contrast to current views (Biddie et al., 2011; Liu et al., 2006), PR binding seems to be favored on binding sequences organized in nucleosomes.

After 1 hr of progestin stimulation, a significant decrease in nucleosome occupancy over the PRbs was observed (Figure 2B and Figure S4D), suggesting nucleosome displacement or remodeling. To measure the magnitude of this change, we calculated the nucleosome-remodeling index (NRI) as the ratio between the nucleosome occupancy before and after hormone administration and ranked all PRbs according to their NRI. A heat map of the of nucleosome occupancy showed high-read density around the majority of the PRbs in basal condition, with a dramatic decrease in signal upon R5020 stimulation (Figure 2C, left panel). Besides, most of the PRbs exhibited high sensitivity to DNase before treatment, and they became even more sensitive after stimulation (Figure 2C, right panel). Representative examples are shown in Figure 2D.

To better understand the relevance of this chromatin remodeling in the PRbs, we analyzed two subsets of sites, corresponding to the top 10% NRI (maximal decrease in nucleosome occupancy after progestin treatment) and bottom 10% NRI (minimal or negligible decrease in nucleosome occupancy after treatment) in more detail. Prior to hormone addition, the top NRI PRbs exhibited high nucleosome occupancy, and the nucleosome signal decreased to basal levels after stimulation (Figure 2E, top panel). These sites overlapped with regions highly enriched in DNase signal in unstimulated cells, which slightly increased upon hormone induction (Figure 2F, top panel). In contrast, low NRI PRbs showed poor enrichment in nucleosomes (Figure 2E, low panel) and in DNase signal (Figure 2F, low panel) both before and after hormone treatment.

The analysis of DNase1-seq data allowed the identification of 58,684 total DHS in untreated cells, of which 69% were maintained after R5020 stimulation. In addition, 48,298 other DHS were generated upon treatment (Figure S4E). A significant proportion of PRbs matched with DHS before treatment (3,812 sites), increasing to 9,647 after induction (Figure S4E). A comparative analysis showed that the majority of the top NRI sites coincided with DHS either before (53%) or after (78%) treatment (Figure 2G, upper panel). Conversely, only 2.5% of the bottom NRI PRbs overlapped with DHS in unstimulated cells, rising to 15% after stimulation (Figure 2G, lower panel).
Figure 2. Genome-wide DNaseI Hypersensitivity and Nucleosome Occupancy in the PRbs
(A) DNaseI hypersensitivity before and after 1 hr R5020 treatment is plotted as the average profile of normalized DNaseI-reads counted around the center of all PRbs.
(B) Nucleosome occupancy before and after 1 hr R5020 treatment is plotted as the average profile of normalized MNaseI-reads counted around the center of all PRbs.
(C) Heat maps of nucleosome occupancy (left) and DNaseI hypersensitivity (right) around the PRbs before and after hormone treatment. (Red, high; black, low.) PRbs (24,436 sites observed at 60 min treatment) are sorted according to the nucleosome remodeling index (NRI) in descending order.

(legend continued on next page)
Genomic Distribution and Properties of PRbs with High and Low NRI

We then evaluated the properties and functionality of the PRbs according to their NRI. Both high and low NRI PRbs were mainly located in intronic and intergenic regions. However, high NRI sites were overrepresented within the −10/+1 kb from TSS (Figure 3A), and they showed higher strength of PR binding (Figure 3B).

A comparative analysis with the gene expression results revealed that the top NRI PRbs were predominantly associated with progestin upregulated genes (Figure 3C). As an additional criterion to evaluate the functionality of the PRbs, we measured their distance to the nearest hormone-regulated gene (Figure 3D). High NRI PRbs accumulated closer to regulated genes with a median distance of 35 kb, as compared with the broader distribution of distances for low NRI PRbs with a median of 101 kb. Together, these results suggest a functional role of high NRI PRbs in progestin-mediated gene regulation. Examples of hormone-responsive genes located in the proximity of high NRI PRbs and of nonresponsive genes close to low NRI PRbs are shown in Figures S5A and S5B, respectively. However, we do not find a good correlation between the level of hormonal induction and the magnitude of the NRI (Figure S6A), suggesting that high nucleosome occupancy and remodeling—and therefore high NRI—is a prerequisite for regulation, but does not determine the extent of transcription, which depends on the site-specific context.

Interestingly, in those transient PRbs detected after 5 min R5020 treatment, but not maintained at later time points, we found low nucleosome enrichment, no decrease in nucleosome occupancy after treatment (Figures 3E and S6B), and low association with induced target genes (Figure 3F). Nucleosome occupancy was also low in late PRbs that appeared only after 6 hr of progestin treatment (Figure S6C).

Taken together, these results support the hypothesis that nucleosome enrichment is necessary for efficient and sustained PR recruitment, which is required to trigger chromatin remodeling and gene regulation. These observations also support the general validity of our previous finding that a positioned nucleosome over the HREs is essential for proper hormonal induction of the MMTV promoter (Chávez and Beato, 1997; Di Croce et al., 1999) and strongly support a positive role of nucleosomes both in PR binding and in hormone stimulation (Vicent et al., 2004).

PRbs at Enhancers and Promoters Exhibit High Nucleosome Occupancy and Remodeling

One difficulty in assigning a functional role to the majority of PRbs is the fact that they are located at large and variable distances from the genes they regulate. In the last few years, high-resolution maps of epigenetic marks and transcriptional regulators have provided insights into the chromatin signatures of regulatory elements (Zentner and Scacheri, 2012; Hon et al., 2009). It was shown that enrichment of monomethylated histone H3 lysine 4 (H3K4me1) is associated with active enhancers, as well as the presence of the histone acetyltransferase p300 and acetylated H3K27 (Heintzman et al., 2007; Visel et al., 2009; Rada-Iglesias et al., 2011). On the other hand, trimethylation of H3 at lysine 4 (H3K4me3) represents a hallmark of promoters.

To get an independent epigenetic criterion about the functionality of the identified PRbs, we performed ChIP-seqs against H3K4me1 and H3K4me3. We found 22,598 sites enriched in H3K4me1 in basal conditions, most of which (90%) were maintained after hormone treatment (Figure S7A). We then considered a PRbs to be promoter-associated when it was in close proximity to a TSS (<1 kb) and overlapped with sites enriched in H3K4me3 (Figure S7B). Likewise, we considered PRbs as associated to enhancers based on the enrichment in H3K4me1 and a distal location from any TSS (≥1 kb up- or downstream). As an additional criterion for enhancer identification, we used ChIP-seq data of p300 in T47D cells obtained from ENCODE (see Figure S7B for details and S7C for validation experiments).

As expected, the promoter-associated PRbs were indeed very close to hormone-regulated genes (median = 490 bp up- or downstream of the TSS of an R5020-responsive gene) (Figure 4A). Epigenetically defined enhancers were significantly enriched around hormone-responsive genes (median = 56 kb for H3K4me1-marked enhancer and 65 kb for those marked by H3K4me1 and p300). PRbs outside of enhancer and promoter regions exhibited a broad distribution of distances to hormone-regulated genes (median = 171 kb).

Moreover, in unstimulated cells, PRbs at promoters were highly enriched in RNA polymerase II (RNAPII), as detected by ChIP-seq (Figure 4B) and on transcribed RNA, as measured by RNA-seq (Figure 4C). In contrast, PRbs over enhancer regions showed low values of RNAPII and of RNA transcripts, indicating weak or absent transcriptional activity (Figures 4B and 4C).

To directly test if distal PRbs have a functional role, we performed a chromosome capture assay (Dekker et al., 2002) on the EGFR gene before and after hormone treatment. Crosslinked chromatin was digested with HindIII and ligated under conditions that favor intramolecular ligation, and the resulting DNA was analyzed with primers over PRbs located in the promoter and a putative distal enhancer 52 kb upstream of the TSS. As shown in Figure 4D, the promoter and the distal PRbs physically interacted only after hormone stimulation, supporting a role of the distal site as a functional enhancer. All of these results indicated that our epigenetically defined regions correspond to actual enhancers and promoters.
Figure 3. Functional PRbs Undergo Nucleosome Remodeling Following Hormone Stimulation

(A) Genomic distribution of PRbs with high and low NRI.

(B) Correlation between NRI and strength of PR binding (average number of PR-reads per PRbs). Inset: boxplot comparing PR-read density (number of reads/bp) in high and low NRI PRbs, p value < 0.0001.

(legend continued on next page)
We then measured the nucleosome occupancy for the PRbs located over enhancers and promoters. PRbs in both of these regions were highly enriched in nucleosomes in unstimulated cells and were strongly remodeled 60 min after hormone treatment (Figure 4E). The remaining PRbs exhibited less nucleosome occupancy and less pronounced remodeling upon stimulation (Figure 4E, last panel). We conclude that PRbs located in functional enhancers and promoters belong to the high NRI class of receptor binding sites.

Chromatin Remodeling at PRbs Is Accompanied by Displacement of Histones H1 and H2A/H2B Dimers

Chromatin remodeling on the MMTV promoter in response to hormone treatment leads to the displacement of linker histone H1 catalyzed by the chromatin remodeling complex NURF, which is recruited to hormone target genes by the activated PR and is required for their hormonal induction (Vicent et al., 2011). To explore this chromatin remodeling genome-wide, we performed ChIP-seq experiments with antibodies to the BPTF subunit of NURF. Following hormone induction we found an enrichment of BPTF over PRbs, particularly in those with high NRI (Figure 5A), in agreement with a possible role of NURF in nucleosome remodeling. A comparison of two sets of 11 PRbs with high and low NRI using ChIP and quantitative PCR (qPCR) showed significant hormone-dependent depletion of histone

Figure 4. PRbs at Enhancers and Promoters Exhibit High Nucleosome Occupancy and Remodeling

(A) Boxplot comparing the distances to the TSS of the closest R5020-regulated gene in the different sets of PRbs. *p value < 0.0001.
(B) RNA polymerase II (RNAPII) occupancy at PRbs. ChIP-seq with an antibody against total RNAPII was performed in unstimulated cells. Figure shows the average profile of RNAPII signal around the different sets of PRbs.
(C) Boxplot comparing the RNA-seq signal around the PRbs located in promoter, enhancer, and nonclassified PRbs.
(D) Interaction of a distal PRbs (putative enhancer H3K4me1+/p300+) with the promoter region in a progestin induced gene, EGFR. Chromosome capture assay (3C) was performed in cells treated 0, 5, or 30 min with R5020. Upper panel: map showing the location of HindIII sites and 3C primers. Lower panel: 3C PCR results obtained on chromatin from cells untreated or treated with R5020, digested with HindIII, and incubated or not with T4 DNA Ligase.
(E) Nucleosome occupancy before and after hormone treatment over the different sets of PRbs.
Figure 5. Chromatin Remodeling around PRbs Is Accompanied by Displacement of Histones H1, H2A, and H2B

(A) Average profile of BPTF signal around the PRbs. ChIP-seq data (Vicent et al., 2011) were used to analyze BPTF occupancy in PRbs with high (left) and low (right) NRI.

(B–E) Histone displacement and Brg1 recruitment in PRbs. Cells untreated or hormone-stimulated were subjected to ChIP assays with antibodies to histones H1 (B), H3 and H4 (C), Brg1 (D), and H2A and H2B (E). A total of 11 PRbs with high NRI (left) and 11 with low NRI (right) were evaluated. Histograms show the quantification of the results by qPCR from two experiments performed in duplicate. Averages and SD are shown. *p value < 0.05.
H1 over PRbs with high NRI (Figure 5B) and almost no changes in the content of H3 or H4 on the same PRbs (Figure 5C). No significant displacement of H1 was observed in an equivalent set of low NRI PRbs (Figure 5B, right panel). These findings suggest that in the subset of nucleosomes occupied by PR and exhibiting high NRI, NURF promotes ATP-dependent displacement of H1, while the nucleosome core particle is not displaced.

Following H1 displacement, hormone treatment leads to BAF complex-mediated displacement of H2A and H2B from the MMTV promoter (Vicent et al., 2009, 2011). In response to R5020 treatment, we consistently found a significant recruitment of the Brg1 subunit of BAF in the set of PRbs with high NRI, whereas no significant recruitment was observed in low NRI PRbs (Figure 5D). Brg1 recruitment was accompanied by displacement of H2A and H2B in PRbs with high NRI, while no significant changes were found over low NRI PRbs, which did not exhibit nucleosome remodeling (Figure 5E). Significant recruitment of BPTF/NURF and BRG1/BAF following hormone treatment was also observed over PRbs epigenetically defined as enhancers (Figures S7D and S7E, respectively), supporting the functional role of these PRbs.

We conclude that displacement of H1 and/or H2A/H2B dimers destabilizes the nucleosome particle, leading to internal cleavage of nucleosomal DNA by MNase and consequent reduction of expected DNA fragment, which is no longer selected for sequencing. This explains the decrease in nucleosome reads over the high NRI PRbs following hormone induction. However, the H3/H4 tetramer is still detectable after hormone treatment, in line with a possible role of the histone tetramer in facilitating hormonal gene regulation (Vicent et al., 2010).

**DISCUSSION**

In recent years, the genomic binding sites for estrogen (ER), glucocorticoid (GR), and androgen receptors (AR) bound with their respective agonist ligands have been identified in several laboratories (Reddy et al., 2009; Welboren et al., 2009; Lin et al., 2009). Progestins have an important role in controlling the biology of breast cancer cells, but in comparison with other steroid hormones the underlying mechanisms are still poorly understood. Our ChIP-seq experiments in the model breast cancer cell line T47D-MTVL identified over 25,000 genomic PRbs after treatment with the agonist progesterin R5020. A global positive correlation was found between the effect of hormones on gene expression and the number and intensity of associated PRbs. Based on the large number of reproducible and stable PRbs found, we defined a consensus sequence for the progesterone response element (PRE), which encompasses several copies of one half of the conventional PRE palindrome and very little conservation in the other half, and is shared by a large majority of the identified PRbs. Recently, 31,457 genomic binding sites for PR bound by the antiprogestin RU486 have been reported (Yin et al., 2012). A comparative analysis with the PRbs here identified shows an overlapping of 63%, indicating that agonist- and antagonist-bound PR may use partially different sets of genomic sites, as previously described for ER (Welboren et al., 2009). Another very recent report compares the genomic binding of PR in two cell lines: T47D and the immortalized normal breast cells AB32 overexpressing PR (Clarke and Graham, 2012). This study identified 6,312 PRbs in T47D, most of them (86%) overlapping with PRbs identified in our ChIP-seq experiments. They also mapped around 8,000 PRbs in AB32 with relatively little overlap with sites identified in T47D, which the author explains by the differential expression of cofactors, such as FoxA1, Ap1, or NF1. However, the lack of overlap may be due to overexpression of recombinant PR or to insufficient coverage, since we have identified 25,000 PR binding sites in T47D cells.

A global inspection of the human genome shows a large number of potential PR binding sequences, of which less than 1% are bound by PR in breast cancer cells. One explanation could be the cooperation of PR with other TFs, as previously proposed for other nuclear receptors. In the case of ER, effective chromatin binding seems to be largely dependent on previous binding of FoxA1 (Carroll et al., 2005; Lupien et al., 2008; Hurtado et al., 2011), which—unlike ER—behaves as a pioneer factor, able to bind nucleosomes (Zaret and Carroll, 2011). However, only a subfraction of the genomic ERbs contain a FoxA1 motif, suggesting that additional factors—possibly including H3K4 methylation—are involved in determining efficient binding (Lupien et al., 2008). As the DNA binding domain of FoxA1 shares the “winged helix” structural motif of linker histones (Clark et al., 1993), FoxA1 could replace histone H1 at sites with receptor binding sequences, locally generating a more open chromatin structure (Cirillo et al., 1998; Taube et al., 2010). Similar results have been reported for AR (Wang et al., 2009), although these results have been recently questioned (Sahu et al., 2011). Although GR can bind to MMTV nucleosomes in vitro (Perlmann and Wrange, 1988; Pfiña et al., 1990; Perlmann, 1992; Li and Wrange, 1993) and in vivo (Truss et al., 1995), recent results suggest that AP1 acts as a pioneer factor for GR genomic binding (Biddle et al., 2011), which occurs preferentially at preformed DNaseI hypersensitive sites (DHS) (John et al., 2008, 2011). In the case of PR, the detected genomic binding sites are enriched for certain TFs (such as Ets, Stat, FoxA1/FoxA2, and AP1), which could contribute to the binding of PR, but none of them was highly significant as needed to explain the large discrepancy between putative binding sites and actually measured PRbs.

We hypothesized that the chromatin organization of potential PREs may determine the accessibility or affinity with which they are recognized by hormone-loaded PR. Controversial results regarding the role of chromatin in hormone-dependent gene activation have been reported. It was shown that on some promoters, such as MMTV, wrapping of the PREs around nucleosomes is required for optimal hormone receptor binding and synergism with other TFs (Di Croce et al., 1999). However, recent studies have indicated that the presence of an open chromatin structure surrounding the hormone receptor binding sites constitutes a prerequisite for receptor binding and subsequent transcriptional regulation (John et al., 2011; Hurtado et al., 2011). These open chromatin regions, pre-established by so-called pioneer factors, such as FoxA1, are assumed to be depleted of nucleosomes (Zaret and Carroll, 2011), although no direct analysis by MNase mapping has been published. Our results in the breast cancer cell line T47D show that prior to
hormone treatment, strong functional PRbs, which are overrepresented around progestin-induced genes, already exhibit high nucleosome occupancy and partly overlap with pre-established DHS, clearly showing that high nucleosome occupancy can occur in DNaseI accessible regions.

Moreover, a fraction of the strong PRbs over nucleosome-rich regions overlap with DHS that only appear following hormone treatment, confirming that PR can initiate chromatin binding and remodeling. Therefore, PR qualifies as a bona fide pioneer factor. This idea has been previously proposed based on studies of PR binding to the MMTV promoter, which contains several PREs organized within a positioned nucleosome (Beato et al., 1995). PR can access PREs of the MMTV promoter in mononucleosomes assembled in vitro (Pña et al., 1990), in minichromosomes assembled in Drosophila embryo extracts (Di Croce et al., 1999), in chromosomes of Saccharomyces cerevisiae (Chávez and Beato, 1997), and when integrated in the genome of cultured breast cancer cells (Truss et al., 1995). Moreover, nucleosomes not only do not interfere with PR binding, but they are also essential for proper hormonal induction of the MMTV promoter (Chávez and Beato, 1997; Di Croce et al., 1999) that requires localized nucleosome remodeling (Vicent et al., 2004).

Upon hormone treatment, nucleosomes containing functional PRbs are remodeled and become more sensitive to cleavage by DNaseI and particularly by MNase. Remodeling is at least in part mediated by NURF and BAF, which remove histones H1 and histone H2A/H2B dimers, respectively. Thus, mechanisms of activation and remodeling observed in the MMTV promoter in the past (Vicent et al., 2004, 2006, 2009, 2011) can now be extended genome-wide.

A classification of the PRbs according to the extent of hormone-induced chromatin remodeling, the nucleosome remodeling index (NRI), shows that sites with a high NRI correspond to strong PRbs, mainly associated with enhancer and promoter regions of progesterone-responsive genes. Very likely these sites correspond to functional PRbs. Nevertheless, we do not find a correlation between the extent of gene regulation and the magnitude of the NRI. Thus, the high NRI is a prerequisite for hormonal regulation but does not by itself determine the level of the transcriptional response, which may depend on the presence of additional TFs or coactivators. In contrast, PRbs that exhibit a low NRI are not associated with hormone target genes and do not recruit chromatin remodelers, suggesting that they are not active as mediators of hormonal gene regulation. It will be interesting to know whether these sites may be functional in other PR-positive cell types. Interestingly, transient PRbs as well as PREs that do not bind PR showed no nucleosome enrichment, no chromatin remodeling after hormone treatment, and no significant association with hormone responsive genes, highlighting the role of nucleosomes acting as preferential binding substrate for PR.

The functional PRbs with a high NRI are enriched over pre-existing DHS that are maintained or even increase upon hormone treatment, whereas nonfunctional PRbs with low NRI are not associated with strong DHS (Figure 2F). We do not know how these DHS are established and maintained, but given that PR is a bona fide pioneer factor, it is possible that they represent the epigenetic memory of past PR binding and activation events. It is also unclear how these DHS are epigenetically marked, although histone methylation, DNA methylation, and H2A.Z (Lupien et al., 2008; Jin et al., 2009) have been postulated to be differentially represented over DHS. The availability of various types of DHS, also those that only appear after hormone treatment, offers us the possibility to study the epigenetic marks involved in their generation and maintenance.

Finally, what could be the reason for the better PR binding and function of PREs included in nucleosomes? We know that PR binds PREs contacting only one side of the DNA double helix and with relatively low affinity compared to other TFs, like NF1, that fully embrace the DNA (Eisfeld et al., 1997). It is possible that PR binding to DNA is not sufficiently stable to orchestrate transcriptional regulation. On the other side, functional PR reaches the target PREs in association with kinases and chromatin remodeling complexes that contact the core and linker histones (Beato and Vicent, 2012). For instance, CDK2 interacts with histone H1, MSK1 with S10 of histone H3, NURF associates with H3K4me3 residue, while BAF interacts with H3K14ac. Therefore, one possibility is that at PREs organized in nucleosomes, binding of PR to DNA is stabilized by interaction of associated factors with histone tails, thus increasing the residence time on chromatin and favoring remodeling and recruitment of coactivators (see model in Figure 6). This hypothesis is supported by our finding that transient PRbs, which are only detected 5 min after hormone treatment and do not persist, exhibit
low nucleosome occupancy (Figure 3E). In addition, cooperation between different PR molecules bound to adjacent or more distant PRES, or between PR and other TFs, could be facilitated in the surface of a core particle or an H3/H4 tetramer particle (Vicent et al., 2010). It is also possible that nucleosomes fulfill an unspecified repressor function in the absence of hormones by making regulatory information unavailable for other TFs binding or by offering histone marks for anchoring of repressive complexes. This will eliminate the need for specific repressors and will make these promoters/enhancers depend on factors like PR, which are able to act as pioneer factors and to recognize target sequences on nucleosomes. Binding of PR with associated remodeling complexes and histone-modifying enzymes will overcome nucleosome repression and modulate binding of either other TFs/coactivators to mediate activation or of corepressors to mediate repression. Thus, in contrast with the currently established view, nucleosomes play a positive role in binding of ligand-activated PR, which recruits ATP-dependent nucleosome remodeling complexes to initiate the opening of chromatin needed for gene regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Treatments

T47D-MTVL human breast cancer cells carrying one stably integrated copy of the luciferase reporter gene driven by the MMTV promoter (Truss et al., 1995) were routinely grown as described (Vicent et al., 2011). Cells were plated in RPMI medium without phenol red, supplemented with 10% dextran coated charcoal-treated FBS. After 48 hr, the medium was replaced by fresh medium without serum. After 24 hr in serum-free conditions, cells were incubated with charcoal-treated FBS. After 48 hr, the medium was replaced by fresh medium and Paro, 1999; Vicent et al., 2011). See Supplemental Experimental Procedures for details.

ChIP and ChIP-Seq

ChIP assays were performed and quantified as previously described (Strutt and Paro, 1999; Vicent et al., 2011). See Supplemental Experimental Procedures for details and list of antibodies.

DNaseI-Seq

DNase hypersensitive sites (DHS) were identified as previously described (Song and Crawford, 2010). Digested DNA was used for deep sequencing using the Solexa Genome Analyzer. See Supplemental Experimental Procedures for details.

Mnase-Seq

Mononucleosomal DNA was prepared as described (Cappabianca et al., 1999). The obtained DNA was purified and subjected to deep sequencing using the Solexa Genome Analyzer. See Supplemental Experimental Procedures for details.

RNA Extraction, RT-PCR, and Expression Arrays

RNA preparation, cDNA synthesis, and quantification of gene products were performed as described (Vicent et al., 2006). See Supplemental Experimental Procedures for details. Global gene expression assays were performed using Agilent Whole Human Gene Expression Microarrays 44K. Three independent samples were analyzed for each treatment. Genes were considered significantly regulated by hormones when expression changed ≥1.5-fold, relative to untreated samples (0 hr) and p < 0.05.

RNA-Seq

RNA was extracted from unstimulated T47D-MTVL cells and submitted to massive sequencing using the Solexa Genome Analyzer. The sequence reads were aligned to the human genome reference hg19, keeping only tags that mapped uniquely with up to two mismatches.

Chromosome Capture Assay

Chromosome capture (3C) assay was performed in T47D-ML cells treated 0, 5, or 30 min with 10 nM R5020. 3C libraries were generated as previously described (Miele et al., 2006) using HindIII as the restriction enzyme. PCR primers sequences are available on request. Primers couple P1/P2 was used to verify comparable efficiencies of digestion and ligation between the different libraries.

ACCESSION NUMBERS

The GEO accession number for the deep sequencing and microarray data reported in this paper is GSE41617

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2012.10.019.

ACKNOWLEDGMENTS

We thank Stefan Dimitrov, Grenoble, for the histone antibodies; Heinz Himmelbauer and the CRG Ultrasounding Facility for help in establishing various sequencing protocols; and Fatima Gebauer, Luciano Di Croce, Juan Valcarcel, and members of the Chromatin and Gene Expression group for help with the manuscript. The experimental work was supported by grants from the Spanish government (BMC 2003-02902 and 2010-15313; CSD2006-00049), the European Union (IP HEROIC), and the Catalan government (AGAUR). L.G. was a recipient of a fellowship from the International PhD program of LaCaixa; G.P.V. was a recipient of a fellowship from the Ramón y Cajal program. We thank the ENCODE Project Consortium for making their data publicly available.

Received: April 9, 2012
Revised: July 31, 2012
Accepted: October 17, 2012
Published online: November 21, 2012

REFERENCES


