A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis

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Recent genomic analyses of transcription factor binding, histone modification, and gene expression have provided a global view of transcriptional regulation by nuclear receptors (NRs) that complements an existing large body of literature on gene-specific studies. The picture emerging from these genomic studies indicates that NRs bind at promoter-proximal and promoter-distal enhancers in conjunction with other transcription factors (e.g., activator protein-1, Sp1 and FOXA1). This binding promotes the recruitment of coregulators that mediate the posttranslational modification of histones at promoters and enhancers. Ultimately, signaling through liganded NRs stimulates changes in the occupancy of RNA polymerase II (Pol II) or the activation of preloaded Pol II at target promoters. Chromosomal looping and/or Pol II tracking may underlie promoter-enhancer communication. Interestingly, the direct target genes of NR signaling represent a limited subset of all the genes regulated by NR ligands, with the rest being regulated through secondary effects. As suggested by previous gene-specific analyses, NR-mediated outcomes are highly cell type- and promoter-specific, highlighting the complexity of transcriptional regulation by NRs and the value of genomic analyses for identifying commonly shared patterns. Overall, NRs share common themes in their patterns of localization and transcriptional regulation across mammalian genomes. In this review, we provide an overview of recent advances in the understanding of NR-mediated transcription garnered from genomic analyses of gene expression, factor localization, and target DNA sequences.

Received November 21st, 2007; Accepted February 7th, 2008; Published February 15th, 2008 | **Abbreviations:** **AcH:** acetylated histones H3/H4; **AcH3K9/14:** H3 acetylated at lysines 9 and 14; **AP-1:** activator protein-1; **AR:** androgen receptor; **CBP:** CREB binding protein; **ChIP:** chromatin immunoprecipitation; **CREB:** cAMP response element binding protein; **DSL:** DNA selection and ligation; **E2:** estradiol; **ERα:** estrogen receptor α; **ERE:** estrogen response elements; **ERR:** estrogen-related receptor; **GR:** glucocorticoid receptor; **H3K4me1:** H3 mono-methylated at lysine 4; **H3K4me2:** H3 di-methylated at lysine 4; **H3K4me3:** H3 tri-methylated at lysine 4; **H3K9me3:** H3 tri-methylated at lysine 9; **H3K79me2:** H3 di-methylated at lysine 79; **NR:** nuclear receptor; **PET:** paired-end tag; **Pol II:** RNA polymerase II; **qPCR:** quantitative real-time polymerase chain reaction; **SAGE:** serial analysis of gene expression; **SRC:** steroid receptor coactivator; **TSS:** transcription start site; **VDR:** vitamin D receptor |

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Introduction

The spatial and temporal regulation of gene expression is an important means by which cells respond to physiological and environmental signals. DNA-binding transcription factors, non-DNA-binding coregulators, and the RNA polymerase II (Pol II) machinery are important for mediating proper (i.e., context-specific or developmentally appropriate) patterns of gene expression [Naar et al., 2001; Orphanides and Reinberg, 2002]. Nuclear receptors (NRs) comprise a superfamily of ligand-regulated, DNA-binding transcription factors, which can both activate and repress gene expression [Mangelsdorf et al., 1995]. Given the number of related factors in the superfamily (49 NR genes and more than 75 NR proteins in mammals; [Robinson-Rechavi et al., 2001]) and their physiological roles throughout the body, NRs make an interesting model to study the mechanisms of transcriptional regulation in response to cellular signals. Transcriptional regulation by NRs is a multistep process involving: (1) the association of NRs with regulatory sites in the genome (i.e., enhancers or silencers) in the context of chromatin, (2) the ligand-dependent recruitment and function of coregulators to modify chromatin and associated factors, (3) the regulation of Pol II binding and activity at target promoters, and (4) the termination or attenuation of NR-dependent signaling [Acevedo and Kraus, 2004; Glass and Rosenfeld, 2000; Kraus and Wong, 2002; McKenna et al., 1999; Metivier et al., 2006]. The complexity of transcriptional regulation by NRs provides many opportunities for exquisite regulatory control of signal-dependent transcriptional responses.

The mechanisms of transcriptional regulation by ligand-bound NRs have been studied extensively in numerous gene-specific studies over the past 30 years. Recently, the development of large-scale genomic methods to analyze gene expression and factor binding to DNA has generated an extensive amount of information about NR-regulated transcription. Some of these methods (i.e., gene expression microarray analyses, ChIP-chip, *in silico* binding site analyses) have been reviewed recently elsewhere [Kim and Ren, 2006; Tavera-Mendoza et al., 2006]. Here, we provide an overview of recent advances in our understanding of NR-mediated transcription focusing on genomic analyses of gene expression, factor localization, and target DNA sequences.

Genomic analyses of NR-regulated gene expression

Gene expression microarrays have been widely used to determine genes whose expression changes upon treatment with NR ligands, such as estrogens, androgens, glucocorticoids, vitamin D3, and lipid metabolites [Frasor et al., 2003; Lee et al., 2003; Quinn et al., 2005; Rogatsky et al., 2003; White, 2004]. Due to the large number of studies available, we will use the regulation of gene expression by ER ligands as an example to illustrate the use of expression microarrays to understand global features of NR-mediated transcription. The approaches and key concepts are similar for most global NR-mediated gene expression studies to date.

How many genes are regulated by estrogen signaling?

The first question addressed by expression microarray analyses of estradiol (E2)-dependent gene expression was "How many genes are regulated by E2 in human cells?" The answer turned out to be less straightforward than expected, and several studies currently available report different numbers of E2-regulated genes, ranging from ~100 to ~1,500 [Carroll and Brown, 2006; Coser et al., 2003; Frasor et al., 2003; Kian Tee et al., 2004; Kininis et al., 2007; Kwon et al., 2007; Levenson et al., 2002; Lin et al., 2004; Lin et al., 2007; Monroe et al., 2003; Rae et al., 2005; Stender et al., 2007] (Table 1). These discrepancies can be attributed to many factors including differences in: (1) the cell lines (tissue origin, ER expression, and growth conditions), (2) the length of the E2 treatment, (3) the microarray platforms and associated experimental variability, and (4) the data analysis protocols. Examples of such types of variation among recent global E2-dependent gene expression analyses are shown in Table 1.

Global features of E2-regulated gene expression

Despite the differences noted above, genomic analyses of E2-regulated gene expression have provided a wealth of useful information for understanding the global features of NR-mediated gene expression. In summary, genomic expression analyses have indicated that: (1) only a limited subset (20-30%) of the E2-regulated genes represent direct E2 targets, as shown by co-treatment with the translation inhibitor cycloheximide [Lin et al., 2004]; (2) the majority of E2-regulated genes after short (1-8 h) hormone treatments are upregulated, whereas most genes regulated after longer treatments (12-48 h) are downregulated [Frasor et al., 2003; Lin et al., 2004]; (3) a surprisingly limited overlap exists between the gene sets regulated by E2 in different cell lines (and even the same cell line grown in different laboratories) [Carroll and Brown, 2006; Frasor et al., 2003; Kininis et al., 2007; Lin et al., 2004; Rae et al., 2005; Stender et al., 2007] (also see Supplemental Table 1 in Kininis et al. [Kininis et al., 2007]); (4) the two subtypes of ER (ERα and ERβ) regulate diverse (>70% different) sets of genes [Kian Tee et al., 2004; Monroe et al., 2003]; (5) selective estrogen receptor modulators (SERMs) have both antagonistic and agonistic effects on global patterns of gene expression, in some cases overlapping with E2-regulated gene sets [Frasor et al., 2004; Levenson et al., 2002]; and (6) some E2-regulated genes are dependent on other DNA-bound transcription factors, such as AP-1, for their expression [DeNardo et al., 2005]. Collectively, the available gene expression microarrays have revealed new global features of NR-mediated transcription, complementing previous gene-specific studies.

Facilitating comparisons between different studies of NR-regulated gene expression

Although it may be unrealistic to expect all researchers in the NR field to follow the same experimental protocols (e.g., RNA processing, microarray platform and handling, and data analysis), means are available to facilitate comparisons among different studies of NR-regulated gene expression. One approach is to evaluate expression microarray performance using complementary approaches, such as Northern blotting or quantitative reverse transcription-PCR, with clear reporting of the confirmation rates [Tan et al., 2003; Taniguchi et al., 2001]. Although this approach would help to control for false positives introduced by the microarray experimental variability, it would not account for false negatives (i.e., genes whose expression changes in the experimental samples, but is not detected by the microarrays). A second simple approach would be to use external controls, such as RNA molecules synthetically produced and spiked in defined amounts to biological samples before hybridization to microarrays [van Bakel and Holstege, 2004]. By using this protocol, differences in microarray sensitivity could be assessed, and proper normalization could be applied to the data. Furthermore, a direct comparison of each study's results to previously published data would greatly facilitate our understanding of the extent and sources of variation. Finally, the deposition of raw (i.e., unprocessed) datasets in databases, such as GEO (http://www.ncbi.nlm.nih.gov/geo/) or NURSA (http://www.nursa.org/), would facilitate meta-analysis efforts aiming to compare and contrast data from different studies using the same analysis algorithms.

Global studies of NR localization

A number of recent studies determined the genomic binding sites of several NRs, including ERα, AR, GR, VDR, ERRα, and ERRβ. Given that most of these studies have focused on the localization of ERα, either to gene promoters, chromosomes, or the whole human genome [Carroll et al., 2005; Carroll et al., 2006; Cheng et al., 2006; Gao et al., 2008; Jin et al., 2004; Kininis et al., 2007; Kwon et al., 2007; Laganiere et al., 2005a; Laganiere et al., 2005b; Lin et al., 2007] (Table 2), we will mainly focus our review on ERα and compare the ERα-specific conclusions to findings about other NRs. The existing studies used a wide variety of chromatin immunoprecipitation (ChIP)-based methods, including combinations of ChIP with microarrays (e.g., ChIP-chip and ChIP-DSL) and with DNA sequencing (e.g.,

Study	Cells	Tissue[a]	ERα expression	E2[b]	Probes on microarray[c]	Thresholds[d]	Regulated genes[e]	Up-regulated	Down-regulated
Frasor *et al.* (2003)	MCF-7	Mammary	Endogenous	4-48 h	~12,000	CS > 12	438	30%	70%
Rae *et al.* (2005)	MCF-7	Mammary	Endogenous	24 h	~12,000	FC > 1.2, P < 0.1	674	N/A	N/A
Carroll *et al.* (2006)	MCF-7	Mammary	Endogenous	3-12 h	~47,000	RMA, Welch t test	1,526	51%	49%
Kwon *et al.* (2007)	MCF-7	Mammary	Endogenous	3-12 h	~47,000	N/A	879	64%	36%
Kininis *et al.* (2007)	MCF-7	Mammary	Endogenous	3 h	~14,500	FC > 2, P < 0.05	217	56%	44%
Lin *et al.* (2007)	MCF-7	Mammary	Endogenous	12-48 h	~47,000	RMA, SAM	1,250	44%	56%
Coser *et al.* (2003)	MCF-7/BUS	Mammary	Endogenous	48 h	~12,000	FC > 2, P < 0.01	730	58%	42%
Lin *et al.* (2004)	T-47D	Mammary	Endogenous	1-24 h	~19,000	FC > 1.2, P < 0.05	386	59%	41%
Rae *et al.* (2005)	T-47D	Mammary	Endogenous	24 h	~12,000	FC > 1.2, P < 0.1	140	N/A	N/A
Rae *et al.* (2005)	BT-474	Mammary	Endogenous	24 h	~12,000	FC > 1.2, P < 0.1	33	N/A	N/A
Levenson *et al.* (2002)	MDA-MB-231	Mammary	Exogenous	24 h	~600	FC > 2	11	N/A	N/A
Stender *et al.* (2007)	MDA-MB-231	Mammary	Exogenous	4-48 h	~12,000	CS > 12	340	N/A	N/A
Monroe *et al.* (2003)	U2OS	Bone	Exogenous	24 h	~6,800	FC > 2	80	85%	15%
Kian Tee *et al.* (2004)	U2OS	Bone	Exogenous	18 h	~12,600	FC > 1.7, P < 0.05	103	65%	35%

[a] Tissue from which the cell line is derived.
[b] Length of E2 treatment. See respective studies for the exact treatment times or time courses.
[c] The number of unique probe sets on the microarray is representative of the number of transcripts assayed in each experiment.
[d] Thresholds used for determining genes regulated by E2. For more details, see the respective studies. CS, confidence score; FC, fold change in expression upon E2 treatment; P, p-value comparing gene expression ±E2 treatment; RMA, robust multi-chip average; SAM, significance analysis of microarrays; N/A, not reported.
[e] Total number of genes regulated by any length of E2 treatment in the study.

Table 1. Microarray analyses of E2-regulated gene expression in human cell lines. Published microarray analyses of E2-mediated gene expression report a variable number of E2-regulated genes. The discrepancies among these studies can be attributed to many factors including differences in (1) the cell lines used, (2) the length of the E2 treatment, (3) the microarray platforms used, and (4) the data analysis protocols used. Examples of these types of variation are indicated.

ChIP-cloning and ChIP-PET) (Table 3), and the results have not always been consistent.

Similarities and differences among the available genomic ERα location analyses

Remarkably, all genomic analyses of ERα localization performed to date in human cells used the same cell line (MCF-7) and most of them used a similar length of E2 treatment (~45 min.) (Table 2). However, the overlap of ERα binding sites among these studies is strikingly limited. For example, only 624 of the 1,234 (50.6%) ChIP-PET ERα binding sites identified by Lin et al., 2007 [Lin et al., 2007] were common to the 3,665 ChIP-chip ERα binding sites identified by Carroll et al., 2006 [Carroll et al., 2006], representing an overlap of less than 20% for the union of ERα binding sites from the two studies. In addition, only one common ERα-bound promoter (TFF1) from the overlapping chromosome 21 and 22 promoter set was identified in two recent ChIP-chip studies, although the same antibody was used [Carroll et al., 2005; Laganiere et al., 2005b; Tavera-Mendoza et al., 2006]. Some of these differences may be attributed to the specific microarray platform used and other experimental variations, but cross-validation of the conflicting results in the available experimental systems is necessary to determine whether the overlap or the union of the identified binding sites represents more accurately the true landscape of ERα targets in MCF-7 cells.

Despite their differences, the ERα localization studies mentioned collectively provide an increasingly clearer view of the general patterns of ERα binding across the genome. For many E2-regulated genes, the ERα binding sites are located at great distances (>100 Kb) from target promoters, while for other E2-regulated genes, the receptor binding sites are located within or near proximal promoter regions [Carroll et al., 2006; Kininis et al., 2007; Kwon et al., 2007; Laganiere et al., 2005b; Lin et al., 2007]. A recent study in mice confirmed the binding of ERα both proximally and distally from promoters *in vivo* [Gao et al., 2008]. Interestingly, genes upregulated by E2 contain more promoter-proximal ERα binding sites than genes downregulated by E2 [Kininis et al., 2007; Kwon et al., 2007; Lin et al., 2007]. Furthermore, genes upregulated by shorter E2 treatments are enriched in ERα binding sites adjacent to their promoters in contrast to genes upregulated by longer E2 treatments, suggesting secondary regulatory effects for the latter gene set [Kininis et al., 2007; Kwon et al., 2007; Lin et al., 2007]. In some cases, ERα-bound distal enhancers were shown to physically interact with the nearest promoters, presumably regulating their E2-dependent expression [Carroll et al., 2005]. The available genomic studies suggest that this long-range regulation may be particularly important for some subsets of genes. Further studies are needed, however, to understand the role of ERα binding to distal enhancers and the generality of the proposed enhancer-promoter looping mechanisms.

Genomic localization themes shared by NRs

In addition to ERα, recent ChIP-chip analyses have examined the localization of AR, GR, VDR, ERRα, and ERRγ at selected regions of the human genome [Bolton

Study	Cells	Method[a]	Microarray[b]	DNA assayed[c]	ERα antibody[d]	-E2[e]	+E2[e]	Data analysis[f]	Binding sites[g]
Jin *et al.* (2004)	MCF-7	ChIP-chip	Custom, PCR-based	~9K CpG islands	HC-20, C-term.	No	24 h	$FE_1 > 2$	70
Carroll *et al.* (2005)	MCF-7	ChIP-chip	Affy, 21mer tiling	Chr. 21 and 22	HC-20, C-term.	No	45 min	$FE_2 > 1.5$, HMM	57
Laganiere *et al.* (2005b)	MCF-7	ChIP-chip	Custom, PCR-based	~19K promoters	HC-20, C-term.	No	45 min	P < 0.005	153
Cheng *et al.* (2006)	MCF-7	ChIP-chip	Custom, PCR-based	~12K CpG islands	D-12, N-term.	Yes	3-24 h	$FE_2 > 2$, SAM	92
Carroll *et al.* (2006)	MCF-7	ChIP-chip	Affy, 21mer tiling	Whole genome	HC-20, C-term.	No	45 min	MP < 10-5, MAT	3,665
Kininis *et al.* (2007)	MCF-7	ChIP-chip	Custom, PCR-based	~900 promoters	Custom, N-term.	Yes	45 min	FC > 1.3, TP < 0.05	47*
Laganiere *et al.* (2005a)	MCF-7	ChIP-clon.	N/A	N/A	HC-20, C-term.	No	N/A	N/A	12
Kwon *et al.* (2007)	MCF-7	ChIP-DSL	Custom, 40mer tiling	~16.2K promoters	HC-20, C-term. H-184, N-term.	Yes	1 h	P < 0.0001, SAM	578
Lin *et al.* (2007)	MCF-7	ChIP-PET	N/A	Whole genome	HC-20, C-term.	No	45 min	PET cluster > 3	1,234

[a] Method used in the study. Clon., cloning; DSL, DNA selection and ligation; PET, paired-end tag. See Table 3. for more details of the methods.
[b] Microarray platform used, when applicable; N/A, not applicable.
[c] The DNA assayed is determined as follows: for ChIP-chip, by the microarray used; for ChIP-DSL, by the size of the DSL oligo pool; for ChIP-cloning and ChIP-PET, by the extent of sequencing performed; N/A, not reported.
[d] The name of the ERα antibody used and the domain it recognizes. C-term., carboxy-terminal; N-term., amino-terminal.
[e] ERα localization was determined in the absence (-E2) or presence of E2 (+E2). For -E2, "Yes" indicates that this condition was tested and "No" indicates that this condition was not tested. For +E2, the length of E2 treatment is shown; N/A, not reported.
[f] A variety of data analysis protocols were used. For details, see the respective studies. FE_1, fold enrichment of IP over preimmune sera; FE_2, fold enrichment of IP over input; HMM, hidden Markov model; P, p-value comparing IP to input, MP, Mann-Whitney U-test p-value comparing IP to input; MAT, model-based analysis of tiling-arrays for ChIP-chip; FC, fold change upon E2 treatment; TP, t-test p-value comparing -E2 to +E2; SAM, significance analysis of microarrays; N/A, not reported.
[g] Binding sites include both promoter-proximal and promoter-distal targets as determined in the presence of E2. * indicates that the binding of ERα to these sites is regulated by E2 (not tested in the other studies).

Table 2. Genomic analyses of ERα localization in human cell lines. Table summarizing the similarities and differences among the genomic analyses of ERα localization in human cell lines published to date.

Method[a]	ChIP processing	Readout assay	Comments
ChIP-chip	Ligate linkers, amplify and fluorescently label fragments	Microarray hybridization	Tiling improves the specificity and accuracy of binding sites detection. Bias introduced by amplification and inability to interrogate repeat-masked regions.
ChIP-DSL	Anneal selected oligos, amplify and fluorescently label fragments	Microarray hybridization	More sensitive, but less cost-efficient than ChIP-chip; Bias introduced by the size of the DSL oligo pool, amplification and the inability to interrogate repeat-masked regions.
ChIP-cloning	Ligate linkers and clone whole ChIP fragments	Sequencing	Unbiased, but large-scale sequencing is needed to distinguish truly enriched sequences from background. Not cost-efficient.
ChIP-SAGE	Ligate linkers, create DNA tags, concatenate and clone tags	Sequencing	Unbiased, interrogates repeat-masked regions, but has lower resolution than related methods and requires extensive sequencing.
ChIP-PET	Clone ChIP fragments, generate, concatenate and clone PETs	Sequencing	Unbiased, localizes binding sites more precisely, interrogates repeat-masked regions, but requires extensive sequencing.
ChIP-Seq	Ligate linkers, amplify and generate clusters	Sequencing	No cloning involved, interrogates repeat-masked regions ,and is more quantitative and cost-efficient than other sequencing-based methods.

[a] Commonly used method names. DSL, DNA selection and ligation; SAGE, serial analysis of gene expression; PET, paired-end tag; Seq, sequencing.

Table 3. ChIP-based methods to study factor localization on a genomic scale. A number of recent studies have determined the genomic binding sites of several NRs. Important technical features, as well as advantages and disadvantages of each method, are summarized.

et al., 2007; Dufour et al., 2007; Pike et al., 2007; So et al., 2007; Wang et al., 2007]. The global features of AR, GR and VDR binding to DNA are remarkably similar to those identified for ERα in that the receptors localize both distally and proximally to gene promoters, and enhancer-promoter looping may be an important mode of transcriptional regulation [Pike et al., 2007; So et al., 2007; Wang et al., 2007]. The ERR location analysis focused on gene promoters and identified a significant overlap in the ERRα- and ERRγ-bound targets, suggesting that the two ERR subtypes may function as heterodimers in human cells [Dufour et al., 2007]. Overall, NRs share some common themes in their patterns of localization across the human genome (i.e., promoter-proximal and promoter-distal binding, long-range enhancer-promoter interactions), although more studies covering a wider range of NRs are needed to fully establish the generality of these patterns.

NR binding sites: lessons from the underlying DNA sequences

Gene-specific studies have shown that NRs can associate with their target DNA sequences through at least two different mechanisms: (1) direct binding to specific response elements as monomers, homodimers, or heterodimers [Klein-Hitpass et al., 1988; Mangelsdorf et al., 1995], and (2) indirect binding (or "tethering") through other classes of DNA-bound transcription factors (e.g., activator protein-1, AP-1) [Kushner et al., 2000]. In addition to directing NRs to specific regions of the genome, the target DNA sequences can also regulate the specificity of NR-mediated responses [Klinge et al., 2004; Kurokawa et al., 1995; Lefstin and Yamamoto, 1998; Loven et al., 2001; O'Lone et al., 2004; So et al., 2007]. Interestingly, although the NR binding sequences may vary extensively from a consensus, the precise sequences at individual NR binding sites are often well conserved among mammalian species [So et al., 2007]. Furthermore, the direct or indirect association of NRs with their target sites may allosterically regulate receptor activity and coregulator recruitment [Shang and Brown, 2002].

Many of the recent genome-wide localization studies have used bioinformatic approaches to search for common motifs in the DNA sequences underlying the identified binding sites. As expected, estrogen-, androgen-, glucocorticoid-, vitamin D3- and estrogen related-response elements (EREs, AREs, GREs, VDREs and ERREs, respectively) were enriched in the binding sites of their cognate NRs [Carroll et al., 2005; Carroll et al., 2006; Cheng et al., 2006; Dufour et al., 2007; Gao et al., 2008; Kininis et al., 2007; Kwon et al., 2007; Laganiere et al., 2005b; Lin et al., 2007; Pike et al., 2007; So et al., 2007; Wang et al., 2007]. Notably, for ERRα and ERRγ, the same consensus ERRE sequence was found for both receptor subtypes, consistent with the binding of these receptors as heterodimers to common sites [Dufour et al., 2007]. In addition to NR response elements, binding elements for various other transcription factors (e.g., AP-1, Sp1, FOXA1, Oct1, CREB, C/EBPα, Myc) were also enriched in NR binding sites [Carroll et al., 2005; Carroll et al., 2006; Cheng et al., 2006; Dufour et al., 2007; Gao et al., 2008; Kininis et al., 2007; Laganiere et al., 2005b; Lin et al., 2007; Wang et al., 2007]. In some cases, the binding elements for the transcription factors were found adjacent to NR response elements (e.g., FOXA1, Myc, SF1, and PAX3 with ERα) [Carroll et al., 2005; Cheng et al., 2006; Laganiere et al., 2005b; Lin et al., 2007], while in other cases, they were found in lieu of NR response elements (e.g., AP-1 with ERα) [Carroll et al., 2006; Kininis et al., 2007]. This latter result provides genomic support for the existence of a tethering mechanism *in vivo*. Gene-specific ChIP assays have confirmed the binding of the transcription factors to their cognate elements, while RNAi-mediated knockdown and mutagenesis of the binding element has demonstrated a role for these transcription factors in ligand-mediated signaling by NRs [Carroll et al., 2005; Carroll et al., 2006; Cheng et al., 2006; Dufour et al., 2007; Kininis et al., 2007; Laganiere et al., 2005b; Lin et al., 2007; Wang et al., 2007]. Collectively, the combination of bioinformatic sequence analyses and experimental validation has greatly extended our understanding of NR binding to DNA and associated crosstalk with other signaling pathways.

Connecting NR binding to gene expression through RNA Pol II

Although the mechanisms of NR binding to cognate binding sites have been well characterized, the underlying mechanisms connecting NR functions to Pol II activity at target promoters are less well understood. Gene-specific studies examining ligand-dependent changes in Pol II occupancy at a limited set of NR-regulated promoters have provided a view of Pol II regulation by NRs that is usually assumed to be true for all NR-regulated genes. In this “classical” view, the binding of NRs and associated coregulators to enhancers ultimately regulates the recruitment of Pol II and general transcription factors to target promoters, thus modulating the expression of the associated genes [Dilworth and Chambon, 2001; McKenna et al., 1999; Metivier et al., 2006; Orphanides et al., 1996] (Figure 1). To examine the generality of this view, several studies have used ligand-treated cells to examine the localization of Pol II to NR-regulated genes using ChIP-chip [Carroll et al., 2006; Kininis et al., 2007; Kwon et al., 2007]. These analyses, summarized in Table 4, have addressed two important questions: (1) where does Pol II bind and (2) how are Pol II binding and activity regulated by NR signaling.

Pol II binding at promoters and distal enhancers

As expected, genome-wide studies showed that in E2-treated cells, Pol II was localized to the proximal promoter regions of most E2-stimulated genes [Carroll et al., 2006; Kininis et al., 2007; Kwon et al., 2007]. In some cases, however, Pol II was also bound to distal ERα enhancers [Carroll et al., 2006; Kininis et al., 2007; Kwon et al., 2007]. Based on gene-specific studies with ER- and AR-regulated genes, two models have been proposed for the actions of Pol II at distal enhancers. In the first, Pol II is thought to track from a promoter-distal

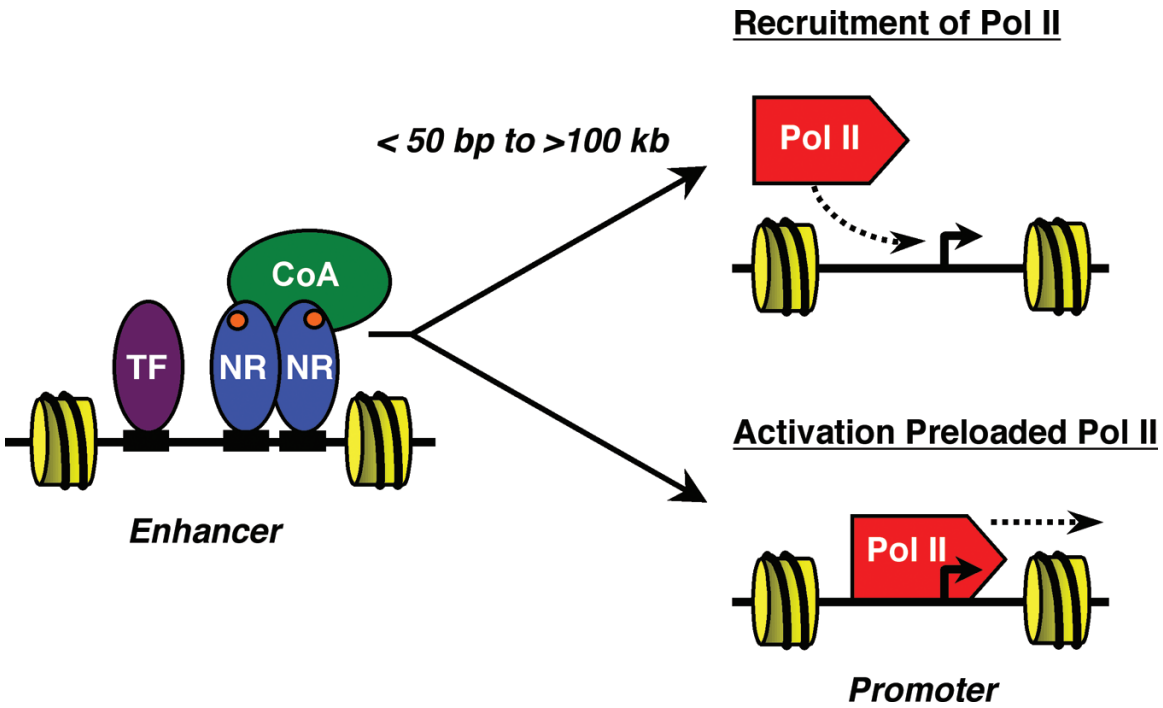

Figure 1. Transcriptional regulation by nuclear receptors. Transcriptional regulation by NRs is a multistep process involving: (1) the binding of liganded NRs to promoter-proximal and promoter-distal enhancers in conjunction with other transcription factors (e.g., AP1, Sp1 and FOXA1), (2) the ligand-dependent recruitment and actions of coregulators (coactivators and corepressors) to modify chromatin and associated factors, and (3) the regulation of Pol II binding or the activation of preloaded Pol II at target promoters.

I. E2-treated cells

Study	Cells	Tissue	Method[a]	Microarray	DNA assayed	Pol II antibody[b]	-E2	+E2	Binding sites[c]
Carroll *et al.* (2006)	MCF-7	Mammary	ChIP-chip	Affy, 21mer tiling	Whole genome	H-224, Rpb1 N-term 4H8, hypo- & hyper-P CTD	No	45 min	3,629
Kwon *et al.* (2007)	MCF-7	Mammary	ChIP-DSL	Custom, 40mer tiling	~18,400 promoters	8WG16, hypo-P CTD	No	1 h	7,963
Kininis *et al.* (2007)	MCF-7	Mammary	ChIP-chip	Custom, PCR-based	~900 promoters	N20, Rpb1 N-term. C21, Rpb1, C-term.	Yes	45 min	56*

II. Steady state cells

Study	Cells	Tissue	Method[a]	Microarray	DNA assayed	Pol II antibody[b]	-E2	+E2	Binding sites[c]
Kim *et al.* (2005)	IMR90	Fibroblast	ChIP-chip	Custom, 50mer tiling	Whole genome	8WG16, hypo-P CTD	Yes	No	9,050
Guenther *et al.* (2007)	hES	Stem cells	ChIP-chip	Agilent, 60mer tiling	~17,350 promoters	8WG16, hypo-P CTD	Yes	No	8,490
Barski *et al.* (2007)	T-cells	Blood	ChIP-Seq	N/A	Whole genome	4H8, hypo- & hyper-P CTD	Yes	No	35,961
Kininis *et al.* (2007)	MCF-7	Mammary	ChIP-chip	Custom, PCR-based	~900 promoters	N20, Rpb1 N-term. C21, Rpb1, C-term.	Yes	45 min	~90

[a] Method used in the study. DSL, DNA selection and ligation; Seq, sequencing.
[b] The name of Pol II antibody used and the subunit/domain that it recognizes. CTD, C-terminal domain; P, phosphorylated.
[c] For Part I, the binding sites identified +E2 are reported. * indicates that Pol II binds in an E2-dependent manner (not tested in the other studies). For Part II, the binding sites identified -E2 are reported.

Table 4. Genomic analyses of RNA polymerase II localization in human cell lines. Table summarizing the similarities and differences among genomic analyses of RNA polymerase II localization in E2-treated (I) and steady-state (II) human cells.

enhancer to the transcriptional start site (TSS) upon treatment with ligand [Louie et al., 2003; Wang et al., 2005]. In the second, enhancer-bound Pol II associates with the TSS through chromosomal looping [Carroll et al., 2005]. Although these suggestions are intriguing, it is difficult to eliminate the possibility that the Pol II detected at distal enhancers results from (1) crosslinking of the enhancers with Pol II-bound promoters (as opposed

to the specific association of Pol II with the enhancer) or (2) the presence of unannotated TSSs throughout the genome [Birney et al., 2007; Hatzis and Talianidis, 2002]. Furthermore, the analysis of Pol II localization only after, but not before, treatment with NR ligands in many of the available studies increases the difficulty in assessing the ligand-dependent effects of NR signaling on Pol II function (see Table 4, Part I).

Regulation of Pol II binding and activity by NR signaling

As noted above, in the “classical” view of NR-regulated transcription, the binding of NRs and coregulators to enhancers regulates the ligand-dependent recruitment of Pol II to target promoters and modulation of gene expression [Dilworth and Chambon, 2001; McKenna et al., 1999; Metivier et al., 2006; Orphanides et al., 1996]. However, recent genomic analyses have indicated that stable Pol II-containing complexes exist at the promoters of many unexpressed genes prior to activation (Table 4, Part II) [Barski et al., 2007; Guenther et al., 2007; Kim et al., 2005; Radonjic et al., 2005]. Accordingly, a recent genomic analysis of Pol II occupancy in both the presence and absence of E2 identified E2-stimulated genes with "preloaded" Pol II at their promoters prior to gene activation [Kininis et al., 2007]. For these genes, E2 signaling stimulates the phosphorylation, but not the recruitment, of Pol II, promoting transcription elongation through the gene rather than transcription initiation. In this regard, NRs, including ERα, have been shown to interact with proteins that regulate Pol II phosphorylation and activity [Aiyar et al., 2004; Kinyamu and Archer, 2007; Wittmann et al., 2005]. Although the generality of this mechanism remains to be determined, it is intriguing to suggest that, in some cases, NRs may control gene expression by regulating the activity, and not the recruitment, of Pol II at target promoters, as shown for other DNA-bound transcription factors [Saunders et al., 2006] (Figure 1).

Defining the direct target genes of NR signaling

As the first genomic analyses of NR-regulated gene expression showed, ligand-dependent regulation of gene expression itself is not sufficient to distinguish between direct and secondary targets of NR signaling. Over the past few years, genomic studies have used various criteria to define the direct target genes of NR signaling, including: (1) regulation of gene expression by short treatments with ligand [Frasor et al., 2003; Lin et al., 2004]; (2) dependence of gene regulation on the receptors, as determined by receptor antagonists and siRNA-mediated knockdown of NRs [Lin et al., 2004; Wang et al., 2004]; (3) identification of NR binding sites at candidate promoters [Kwon et al., 2007; Lin et al., 2007]; (4) loss of ligand-regulated gene expression after co-treatment with protein translation inhibitors [Lin et al., 2004; Rogatsky et al., 2003]; and (5) evidence for changes in Pol II promoter occupancy (an early step in transcriptional regulation) prior to changes in mRNA accumulation [Kininis et al., 2007]. Although each of the above criteria alone is not sufficient, their use in combination can provide a powerful means for determining the direct genomic effects of NRs and their ligands on gene expression.

Genomic analyses of coregulator localization and chromatin modifications

After binding to their genomic targets, NRs mediate their ligand-dependent actions by recruiting positive or negative coregulators. These coregulators, which are shared by various other DNA-binding transcription factors, include: (1) histone- and factor-modifying enzymes, such as the histone acetyltransferase p300 and the histone demethylase LSD1, and (2) bridging factors, such as the steroid receptor coactivators (SRCs), which function to recruit the histone- and factor-modifying enzymes to ligand-bound receptors [Jepsen and Rosenfeld, 2002; Lonard and O'Malley, 2006; McKenna et al., 1999; Perissi and Rosenfeld, 2005]. Although the ligand-dependent recruitment and release of NR coregulators has been examined in detail for a number of genes, the localization of NR coregulators on a global scale has not been studied extensively.

The available genomic localization studies for NR coregulators, including SRCs, p300/CBP, and LSD1, have provided an initial view of the global function of these factors with respect to NR-dependent signaling. All of the aforementioned NR coregulators were found to bind to both promoter-proximal and promoter-distal regions, mirroring the binding patterns observed for NRs [Gamble and Kraus, 2007; Garcia-Bassets et al., 2007; Heintzman et al., 2007; Kininis et al., 2007; Kwon et al., 2007; Labhart et al., 2005]. In recent studies, nearly all of the SRC-bound sites were also occupied by ERα, suggesting a strong link between the recruitment of coregulators and the binding of NRs to sites across the genome [Kininis et al., 2007; Labhart et al., 2005]. A given NR coregulator, however, is not located at all NR binding sites, suggesting that NRs recruit a variety of coregulators in a target gene-specific manner [Garcia-Bassets et al., 2007; Kininis et al., 2007]. Accordingly, the correlation between ERα and SRC recruitment held only for E2-stimulated genes, and no SRC was detected at the promoters of E2-repressed genes [Kininis et al., 2007]. Further studies are necessary to understand the mechanisms regulating the selective recruitment of coregulators to some, but not other, NR binding sites in the same cell.

In addition to studying the localization of histone-modifying coregulators, several studies have examined the state of chromatin, as indicated by histone modifications, at promoters of NR-regulated genes. Histone modifications, such as acetylation and methylation, can regulate NR-mediated transcription by creating (1) a chromatin landscape more or less favorable to gene expression and (2) binding sites on histones for regulatory proteins, with specific modifications specifying transcriptional activation or transcriptional repression [Fischle et al., 2003; Jenuwein and Allis, 2001; Seet et al., 2006; Shogren-Knaak et al., 2006]. Acetylation of histones, primarily H3 acetylated at lysines 9 and 14 (AcH3K9/14),

was observed at both promoter-proximal and promoter-distal ERα binding enhancers [Kininis et al., 2007; Kwon et al., 2007]. The ligand-dependent changes in histone acetylation at the promoters of E2-stimulated or -repressed genes correlated with Pol II recruitment or release, respectively [Kininis et al., 2007]. In contrast to histone acetylation, histone methylation showed a more complicated pattern. While some histone methylation marks for active genes (e.g., H3 mono- and di-methylated at lysine 4 ; H3K4me1 and H3K4me2) were detected at both promoter-proximal and promoter-distal enhancers, other marks for active genes (e.g., H3 tri-methylated at lysine 4; H3K4me3) were found exclusively at promoter-proximal NR binding sites [Kwon et al., 2007], as reported for other DNA-bound transcription factors [Barski et al., 2007; Guenther et al., 2007; Heintzman et al., 2007]. Furthermore, histone methylation marks previously associated with inactive genes (e.g., H3 tri-methylated at lysine 9 and H3 di-methylated at lysine 79; H3K9me3 and H3K79me2, respectively) were enriched in some NR-regulated genes after gene activation, suggesting that the role of these modifications is more complicated than previously thought [Kwon et al., 2007]. Interestingly, the ratio of AcH3K9 to H3K9me2 at promoters was shown to be a good marker for ERα recruitment and E2-dependent regulation of gene expression [Cheng et al., 2006]. Collectively, these results suggest that NR signaling regulates the chromatin state of its genomic targets through histone modification. Certain modifications, however, have distinct gene-specific roles and their effects on gene expression cannot be generalized [Berger, 2007].

Summary and future perspectives

Recent genomic analyses of transcription factor binding, histone modification, and gene expression have provided a global view of transcriptional regulation by NRs, which complements the existing literature of gene-specific studies. These genomic analyses have revealed some common themes for the molecular regulation of gene expression by NRs. These themes include: (1) direct or indirect binding of NRs at promoter-proximal and promoter-distal enhancers in conjunction with other transcription factors (e.g., AP-1, Sp1 and FOXA1), (2) NR-dependent recruitment of coregulators and subsequent modification of histones at both enhancers and promoters, and (3) ultimately, NR-dependent regulation of the recruitment of Pol II or the activity of preloaded Pol II at the promoters of target genes. This regulation may be facilitated by chromosomal looping and Pol II tracking, which can promote the enhancer-promoter communication. Overall, the direct targets of NR signaling seem to be limited to a subset of all the genes regulated by NR ligands, with the rest likely to be regulated through secondary effects. In addition, NR-dependent gene regulation occurs in a highly cell type- and promoter-specific manner, indicating the complexity of transcriptional regulation by NRs and the value of genomic analyses in identifying commonly shared patterns. Despite their differences, NRs share common themes in their patterns of localization and transcriptional regulation in the human genome. Further studies are needed to confirm the generality of these patterns and examine the associated mechanisms in more detail.

Although the global picture of NR-mediated transcription provided by the studies described herein has advanced our understanding of NR-dependent transcription, many questions remain unanswered and new ones have emerged from the wealth of available genomic data. One of the key challenges in the NR field today is to determine the *in vivo* functionality of NR binding sites that have been identified in genomic analyses, especially with respect to enhancer-promoter interactions and cell type-specificity. A common theme from the numerous available chromatin- and transcription-related genome-wide factor localization analyses is that the binding of a factor to a specific site in the genome does not always correlate in an obvious way with a functional outcome for a specific target gene (as determined, for example, by RNAi-mediated knockdown of the relevant factor, coupled with gene-specific expression analyses). In some cases, a particular binding event may only have a detectable functional outcome in a particular cell type. Thus, validation of the functionality of individual NR binding sites *in vivo* is essential (e.g., by mutating the associated DNA elements in their native genomic and chromatin environment and examining the outcome in cells and animals).

A number of other areas of NR biology will benefit from additional genomic analyses. For example, ongoing efforts to map long-range interactions between distal NR binding sites and target promoters on a genomic scale should greatly facilitate our understanding of target genes regulated by NR binding. Moreover, additional genomic studies of NR coactivator and corepressor localization will help to provide a broader picture of the ligand-dependent effects of NR binding to genomic sites. Furthermore, genomic analyses of factors involved in the tethering of NRs to target enhancers (e.g., such as AP-1 and NF-κB) will provide new insights into this poorly understood, but physiologically important, mechanism. Finally, additional global analyses of Pol II localization and activity are needed to determine the generality of Pol II recruitment and Pol II activation at NR-regulated genes. Collectively, genomic analyses of gene expression, factor localization, and target DNA sequences provide a new set of tools for examining the generality of previously studied mechanisms, as well as identifying new mechanisms by which NRs mediate their physiologically important actions.

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