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Estrogen Receptor-α Directs Ordered, Cyclical, and Combinatorial Recruitment of Cofactors on a Natural Target Promoter

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Summary

Transcriptional activation of a gene involves an orchestrated recruitment of components of the basal transcription machinery and intermediate factors, concomitant with an alteration in local chromatin structure generated by posttranslational modifications of histone tails and nucleosome remodeling. We provide here a comprehensive picture of events resulting in transcriptional activation of a gene, through evaluating the estrogen receptor-α (NR3A1) target pS2 gene promoter in MCF-7 cells. This description integrates chromatin remodeling with a kinetic evaluation of cyclical networks of association of 46 transcription factors with the promoter, as determined by chromatin immunoprecipitation assays. We define the concept of a "transcriptional clock" that directs and achieves the sequential and combinatorial assembly of a transcriptionally productive complex on a promoter. Furthermore, the unanticipated findings of key roles for histone deacetylases and nucleosome-remodeling complexes in limiting transcription implies that transcriptional activation is a cyclical process that requires both activating and repressive epigenetic processes.

Introduction

Estrogens have pivotal functions in both female and male physiology. In particular, 17β-estradiol (E_2) has a central role in the proliferation and differentiation of responsive cells, through changing the expression profile of target genes within responsive tissues (Feigelson and Henderson, 1996; Nilsson et al., 2001). The effects of E_2 are mediated through binding to transcription factors belonging to the nuclear receptor (NR) superfamily, the estrogen receptors (ERα and ERβ). ERs activate transcription through associating with estrogen-responsive elements (ERE) located within the promoter regions of target genes (Robinson-Rechavi et al., 2003). However, transcription is achieved within a restrictive environment, as the packaging of promoter DNA and histones into nucleosomes precludes gene expression (Wolffe, 1992; Dillon and Festenstein, 2002). The specific and ordered recruitment and assembly of several multisubunit protein complexes on promoters provides chromatin with the plasticity required for transcription initiation (Cosma, 2002; Narlikar et al., 2002). Accordingly, ERα undergoes major structural rearrangements on association of ligand to expose binding surfaces that recruit transcription cofactors (Brzozowski et al., 1997). In the last 10 years, a plethora of cofactors involved in ER-mediated transactivation have been identified (Klinge, 2000; McKenna and O'Malley, 2002; Belandia and Parker, 2003). Among these proteins are the SWI/SNF complexes (Belandia et al., 2002) that alter the spatial organization of nucleosomes in an ATP-dependent manner (Kassabov et al., 2003). Other enzymes recruited by ER modify the lysine or arginine residues of histone tails (Lee et al., 2001), processes necessary for chromatin remodeling (Berger, 2002; Marmorstein, 2001). Histones acetyl-transferases (HAT) conscripted by ER include members of the p160 subfamily such as SRC1 (Onate et al., 1995) or the integrator complexes p300/CBP and p/CAF (Lee et al., 2001). Histone methyl-transferase (HMT) activity, provided by proteins such as CARM1 or PRMT1, also poise a promoter for transcription through methylation of arginine groups within histones (Marmorstein, 2001). Consequently, the nucleosomal architecture of estrogen-responsive promoters is modified following ER binding and subsequent recruitment of protein complexes (Nye et al., 2002).

Induction of transcription requires the formation of the preinitiation complex (PIC), which comprises the six $TF_{II}A$ to F complexes and RNA polymerase II (Pol II; Berk, 1999) on the promoter. Following many years of investigation, a model emerged (Orphanides, et al., 1996; Ogbourne and Antalis, 1998; Emerson, 2002) postulating that recruitment of TBP, a subunit of $TF_{II}D$ that binds the TATA box, first becomes stabilized by $TF_{II}A$. $TF_{II}B$ next joins the complex, assisting in the selection of the initiation site, followed by RNA Pol II once the recruitment of $TF_{II}B$ has structurally remodeled the PIC (process of isomerization). Subsequent initiation of transcription involves recruitment and structural remodeling of the TRAP/mediator complex, which stimulates phosphorylation of the largest subunit of Pol II (Rbp1, or CTD) by $TF_{II}H$ (Malik and Roeder, 2000; Davis et al., 2002; Woychik and Hampsey, 2002). This event provokes exchange of mediator by elongator complexes (Otero et al., 1999), thereby allowing transcription to initiate. ER is known to contact $TF_{II}B$, $TF_{II}E$, and $TF_{II}F$ and subunits of $TF_{II}D$ (TAFs and the TBP; Sabbah et al., 1998; Wu et al., 1999), providing mechanistic evidence for a direct role for ER in transcriptional activation.

Apart from the sequential recruitment of HATs and TRAP/mediator complexes on E_2-responsive genes (Shang et al., 2000, Burakov et al., 2002), no experimental data yet describe the chronological sequence of events that occur during the initiation of transcription. Further, responses to E_2 have to be constrained in their duration, enabling cells to sense E_2, respond, and then

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stop responding to E_2. Although remodeling complexes and proteasome-mediated degradation may be involved in this obligatory limit to gene expression (Lonard et al., 2000; Fletcher et al., 2002; Reid et al., 2003), no complete experimental description of this process has yet been obtained. Through the use of chromatin immunoprecipitation (ChIP) assays, we provide an extensive description of the cyclic events that occur to initially achieve and subsequently limit transcription mediated by human ERα (hERα) on an endogenous estrogen-responsive promoter. These results are integrated into a model of transcription activation that considers cycles of protein recruitment on a promoter in vivo, and the prospective generalization of the underlying notions raised by this analysis is then discussed.

Results

pS2 Gene Expression Requires hERα

A general model system where hERα is the key regulatory element was required to elucidate the dynamics of a gene activation by hERα. Among the genes tested, we observed that a 3 hr treatment with 10 nM estradiol (E_2) induces the endogenous estrogen-inducible pS2 gene (Masiakowski et al., 1982) mRNA levels 12-fold in hERα positive human MCF7 breast cancer cells. Moreover, although the pS2 gene is not transcribed in hERα negative MDA-MB231 cells, reexpression of hERα in these cells (MDA::hERα) induces a ligand dependent pS2 expression phenotype (Figure 1A). Expression of the pS2 gene in MCF7 and MDA::hERα cells parallels the binding of hERα and activated/phosphorylated RNA polymerase II (P-Pol II) on the pS2 gene promoter, as assessed in chromatin immunoprecipitation (ChIP) assays (Figure 1B). In addition, in cells expressing hERα but not in MDA-MB231 cells, the pS2 promoter was found to contain acetylated histones, a marker of an active transcription locus (Berger, 2002; Figure 1B). Together, these results indicate that hERα is the key transcriptional factor that poises the pS2 gene for expression and fulfills our requirements for a model promoter.

Identification of Factors Recruited to the pS2 Promoter

Intending to comprehensively describe events initiated by hERα involved in the modulation of pS2 gene activity, we selected target proteins for evaluation through ChIP assays by a thorough bibliographic screening. Criteria for significance were: (1) a physical interaction with hERα or another NR; and (2) a described involvement in transcription. The rationale for consideration of these 46 different proteins is shown in the Supplemental Tables S1 and S2 available at http://www.cell.com/cgi/content/full/115/6/751/DC1. ChIP patterns obtained on chromatin prepared from MCF-7 cells, treated 3 hr with 10 nM E_2 after 3 days of steroid-deprivation, demonstrate that a number of proteins are engaged on the pS2 promoter (Figure 1C). Importantly, proteins present on the pS2 promoter before treatment with E_2 include basal transcriptional factors, active Pol II, certain histone acetyl-transferases (HAT) and histone methyl-transferases (HMT). This basal transcriptional activity of the pS2 gene implies that steroid deprivation of the MCF-7 cells is not sufficient to achieve complete silencing of the pS2 gene.

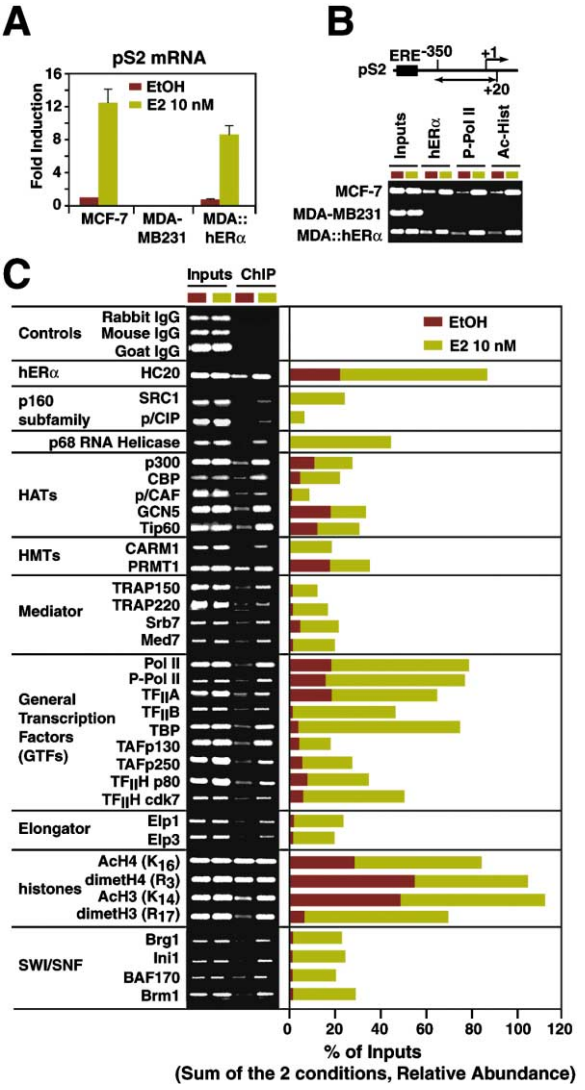

Figure 1. In Vivo Identification of the Transcription Factors Involved in pS2 Gene Activity

(A) The expression of the pS2 gene was monitored by real-time PCR on reverse-transcribed mRNA from hERα positive (MCF-7) or negative (MDA-MB231) cells or MDA-MB231 cells stably expressing hERα. After 52 hours of culture in stripped-media, cells were treated for 3 hr with 10 nM Estradiol (E_2) or ethanol (EtOH) as vehicle control. The pS2 mRNA levels were normalized against invariant GAPDH mRNA, as measured by real-time PCRs.

(B) Chromatin immunoprecipitations (ChIP) determining the recruitment of hERα, Phosphorylated Pol II (P-Pol II) and acetylated histones (Ac-Hist) to the pS2 promoter after 3 hr treatment with 10 nM E_2 or EtOH.

(C) ChIP experiments performed using chromatin prepared from untreated or 2 hr 10 nM E_2-treated cells, quantified by real-time PCR (values in the right hand image).

However, these experiments clearly demonstrate that proteins targeted to the pS2 promoter after E_2 treatment include known cofactors of hERα [p160 such as SRC1 (Onate et al., 1995) and the p68 RNA helicase (p68, Endoh et al., 1999)], ATP-dependent chromatin remodeling complexes (SWI/SNF), HATs and HMTs, components of the TRAP/mediator complexes and basal transcriptional factors. Subsequent experiments assessed the kinetics and organization of the recruitment of these proteins.

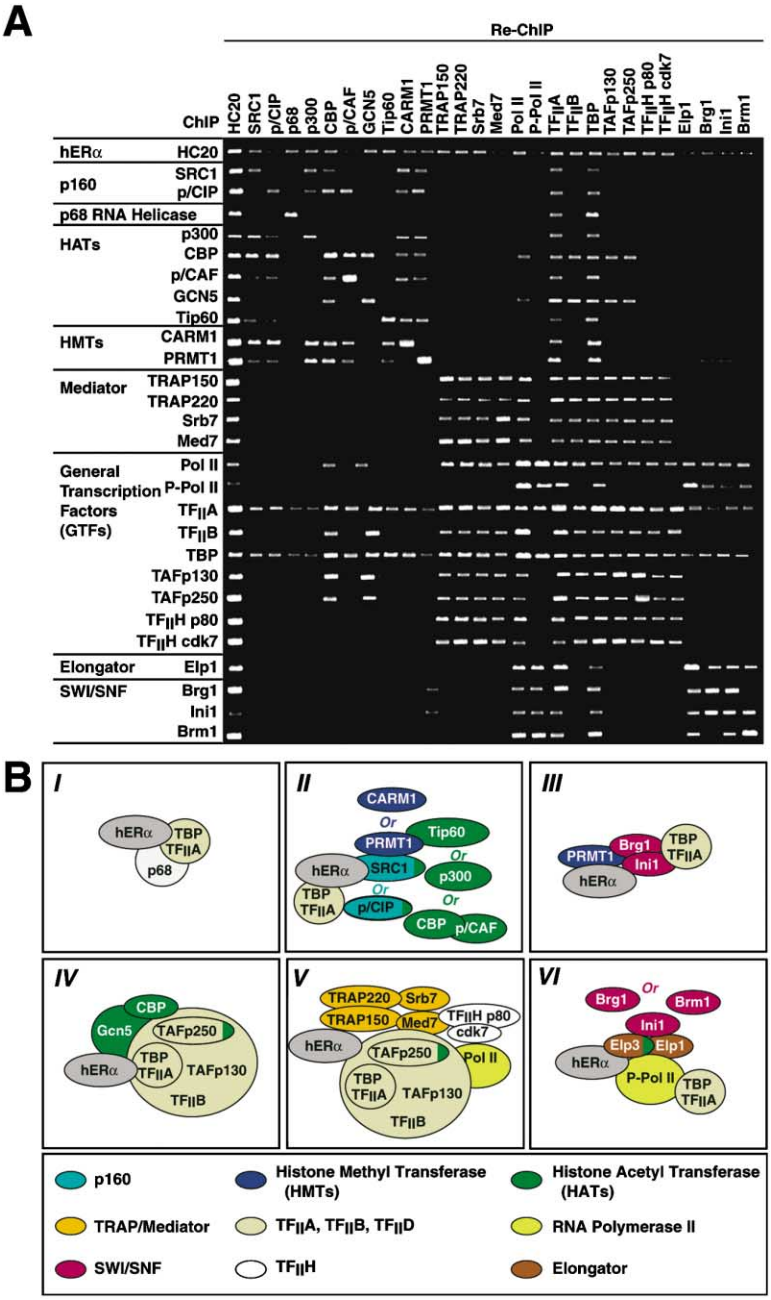

Figure 2. Re-ChIP Screening of the Factors Recruited to the pS2 Promoter

(A) Chromatin prepared from cells treated for 3 hr with 10 nM Estradiol (E_2) was subjected to the ChIP procedure with the antibodies shown at the left side, and again immunoprecipitated using antibodies shown at the top of the image.

(B) Scheme illustrating the complexes indicated through the comprehensive Re-ChIP analysis of transcription factors on the pS2 promoter.

Dissecting the Protein Complexes Involved in pS2 Gene Activation

As seen above, at least 30 different proteins become engaged on the pS2 promoter in the presence of E_2. In order to identify the topology and combinatorial nature of these corecruitments, we used re-ChIP assays to establish which alternative combinations of factors are present on the pS2 promoter (Figure 2A). HATs, p160, components of SWI/SNF and HMTs are combinatorially recruited, as neither SRC1 and p/CIP, nor CARM1 and PRMT1 are ever found on the same set of pS2 promoters. These alternative possibilities likely reflect functional redundancy among these proteins. PRMT1 associates with Brg1 but never with Brm1, which are alternative ATPases of the SWI/SNF complex (Kadam and Emerson, 2003). Similarly, CBP, but not p300 or Tip60, is the HAT associated with Pol II on the pS2 promoter. In contrast to the HATs and HMTs that are present in multisubunit complexes with $TF_{II}A$ and TBP, the p68 RNA helicase (p68) is found alone with these two transcription factors. Importantly, TRAP/mediator is the only complex associated with $TF_{II}H$, presumably reflecting the role mediator has in stimulating the phosphorylation of Pol II by cdk7, a component of $TF_{II}H$ (Kim et al., 1994). The conclusions of this analysis is shown in Figure 2B, depicting six different complexes of proteins present exclusively on the pS2 promoter: i) one that is p68 dependent; ii) a complex containing alternative HATs, p160 and HMTs; iii) a specific PRMT1/SWI/SNF complex; iv) a GCN5 dependent pathway; v) a mediator/$TF_{II}H$ dependent path-

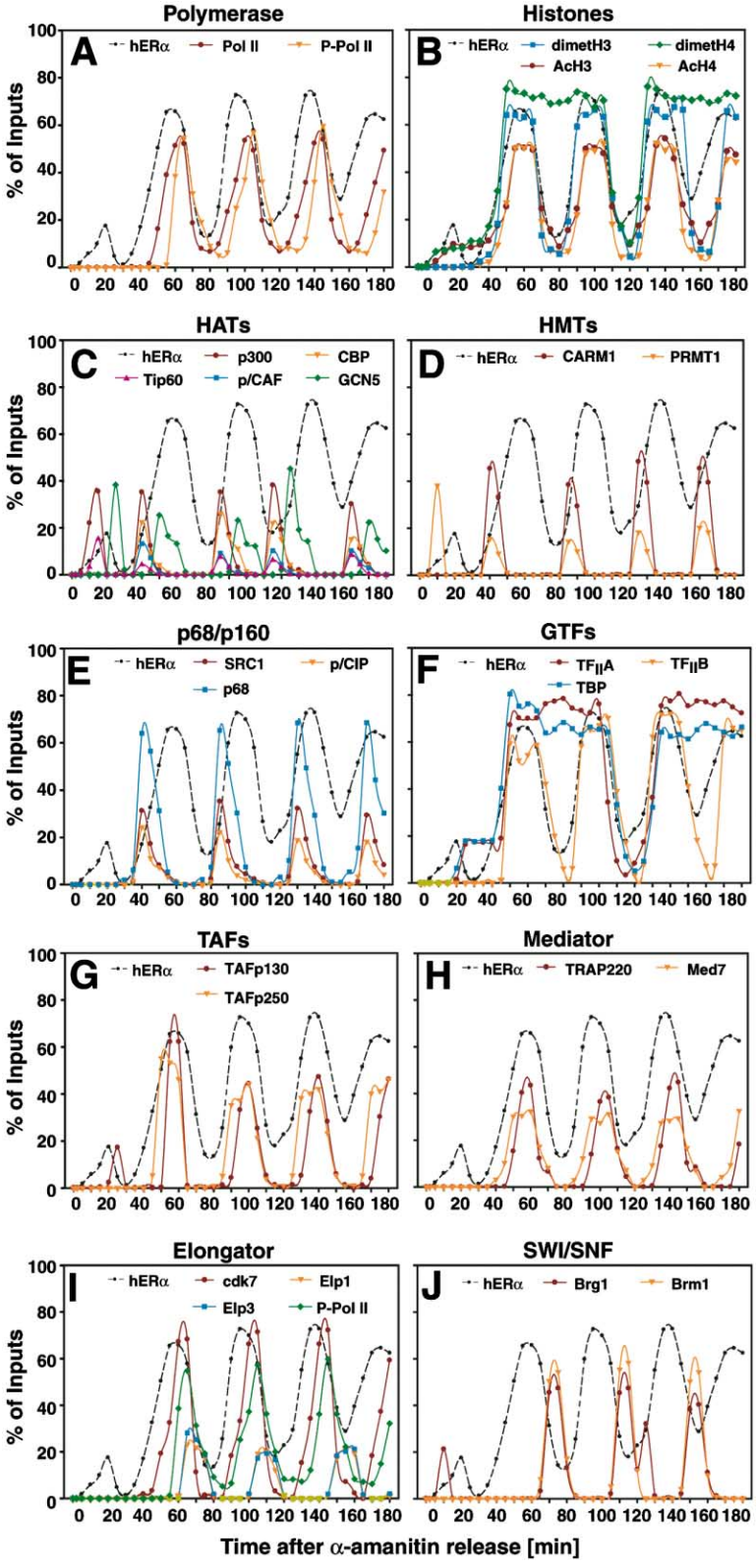

Figure 3. Dynamics of Cofactor Recruitment Directed by E_2-Liganded hERα on the pS2 Promoter

Kinetic ChIP experiments were performed using specified antibodies as shown within the images. After 2 hr treatment with 2.5 μM α-amanitin, cells were washed and placed in media supplemented with 2.5% dextran-charcoal treated FCS including 10 nM Estradiol (E_2). Then, chromatin was prepared on sampled cells at 5 minutes intervals. The amount of immunoprecipitated pS2 promoter was quantified by real-time PCR. Values, expressed as % of the inputs, are the mean of three separate experiments, and have a SD < 2%. All ChIP were performed from a single chromatin preparation for each time point.

way; and vi) an activated Pol II specific network of proteins including elongator and SWI/SNF. These data indicate that alternative protein complexes are sequentially formed on the pS2 promoter, which is further explored below.

Cyclical Recruitment of Transcription Factors on the pS2 Promoter

A clear assessment of the kinetics of recruitment of the proteins was prevented by the presence of general transcription factors on the pS2 promoter in the absence

of E_2 (Figure 1C), responsible for the basal expression of the pS2 gene (Figure 1A). To circumvent this limitation, we abrogated the background activity of the pS2 promoter by cultivating MCF-7 cells for 3 days in steroid-free media followed by treatment with 2.5 μM α-amanitin for 2 hr. This generates a synchronized cell population in which the pS2 promoters are devoid of transactivating factors and whose local histones are not acetylated (Shang et al., 2000; Reid et al., 2003; Supplemental Figure S3 available on *Cell* website). Following α-amanitin synchronization, removal through washing followed by the addition of 10 nM E_2, endogenous pS2 promoters become cyclically permissive to the binding of E_2-liganded hERα with a frequency of 40–45 min, following an initial cycle of 20 min (Figure 3A). Although Pol II is not recruited during the first cycle, afterward it lags behind hERα association by 10 min. Phosphorylation of Pol II, required for transcriptional competence, then requires a further 10 min, as judged by the use of a specific antibody directed against the phosphorylated form of Pol II. During the initial nonproductive cycle, histones H3 K_{14} and H4 R_3 become acetylated and dimethylated to steady-state levels (Figure 3B), at periods when Tip60, p300, and PRMT1, but not the others HATs and HMTs, become recruited (Figures 3C, 3D, and 3E). This demonstrates a requirement not only for the enzymatic activities of these proteins but for these specific factors in inducing transcriptional competence within the pS2 promoter organization. Further, these data confirm the substrate specificity of PRMT1, which is known to methylate H4 R_3 but not H3 K_{14} (Wang et al., 2001a).

After the first transcriptionally silent cycle, the levels of pS2 promoter bound to modified histones increase dramatically (Figure 3B), following the mobilization of the HATs and HMTs (Figure 3C). However, while local histones on the pS2 promoter become modified by acetylation on H4 K_{16}, H3 K_{14} and dimethylation on H3 R_{17}, coincident with hERα cycles, the occurrence of dimethylation on H4 R_3 corresponds to that for TBP and $TF_{II}A$ (Figures 3B and 3F). While $TF_{II}B$, which directs isomerization of the preinitiation complex (PIC), becomes cyclically engaged in every cycle of hERα, remarkably, TBP and $TF_{II}A$ associate with the pS2 promoter through a double cycle whose initiation is coincident with greater levels of GCN5 association (40% as compared to 22%, Figures 3C and 3F). This suggests that these two transcription factors help in GCN5 enrollment, and that GCN5 plays distinct roles to other HATs, as its recruitment is slightly delayed compared to other HATs. Importantly, the TBP associated factors TAF130 and TAF250, both components of the $TF_{II}D$ multimeric complex, are recruited to the pS2 promoter with kinetics different than TBP (Figure 3G). This suggests that $TF_{II}D$ is formed, or at least completed, on the promoter with TAFs 130 and 250. Recruitment of TRAP/mediator, cdk7, apparition of a P-Pol II signal followed by elongator occurs sequentially and cyclically (Figures 3H and 3I) as a consequence of hERα driven cycles.

The Re-ChIP data (Figure 2), in conjunction with the kinetics of association of p160 coactivators, HATs and HMTs on the pS2 promoter (Figures 3C, 3D, and 3E), point to alternative combinatorial recruitments for (1) p300, CBP or Tip60; (2) PRMT1 or CARM1; (3) SRC1 or p/CIP; and (4) the alternative ATPases Brg1 and Brm1 of the SWI/SNF complex that associates when the pS2 promoter becomes refractory to hERα (Figure 3J).

Dynamics of hERα-Mediated Assembly of a Competent PIC

Three different types of cycle of protein recruitment to the pS2 promoter occur in the presence of E_2. These are an initial transcriptionally unproductive cycle followed by two different transcriptionally productive cycles, exemplified through comparison of the cycles between 30 to 80 and between 120 to 160 min. These differ in the proteins present on the promoter at their start: at 120 min, the pS2 promoter does not include modified histones nor TBP and $TF_{II}A$. To provide further detail on the sequence of events involved in the different cycles, we performed ChIP kinetics at one-minute resolution.

During the first transcriptionally unproductive cycle, association of hERα with the pS2 promoter was found to be biphasic (Figure 4A). The first protein recruited after hERα during the initial unproductive cycle is Brg1, followed by PRMT1, p300, or Tip60, then by TBP, $TF_{II}A$, and TAF130, and finally GCN5 (Figures 4A and 4B). In the next two transcriptionally productive cycles, which are identical to each other, p68 is initially engaged on the pS2 promoter (Figures 4C, 4D, and Supplemental Figure S4 available on *Cell* website), followed by HMTs and then by SRC1 (or p/CIP, data not shown) while other HATs associate later. In the third cycle type (exemplified by data acquired between 120 and 160 min), the sequence of protein recruitment relies on an initial action of SWI/SNF (Brg1 but not Brm1), followed by p68 and the mobilization of the p160, HATs, and HMTs, leading to acetylation and dimethylation of the histones H3 and H4 (Figures 4E and 4F). The subsequent association of TBP and $TF_{II}A$ then generates a situation similar to the one observed in the other transcriptionally productive cycle (30–80 min). The Figure 4G illustrates the common sequence of recruitment occurring in both transcriptionally productive cycles: in essence, following the actions of the HATs and HMTs, the pS2 promoter engages TAFs, GCN5, and $TF_{II}B$, facilitatilng recruitment of Pol II, which is then activated by the cdk7 kinase of $TF_{II}H$ (Berk, 1999), allowing the association of elongator proteins.

Cyclic Inactivation of pS2 Promoter Transcriptional Competence

We next examined mechanisms that act to remove hERα and other transcription factors from the pS2 promoter. We were aware that: (1) SWI/SNF complexes engage the promoter at the onset of "clearance" (Figure 3) and that (2) deacetylation of histones commences at this point (Figure 3). We therefore evaluated the recruitment of histone deacetylases (HDACs) and nuclear receptor corepressors, NCoR or SMRT, which bridge HDACs to nuclear receptors (Lee et al., 2001). HDACs1 and 7, but not HDAC3, NCoR, or SMRT, were found to associate with the pS2 promoter in the presence of E_2 (Figure 5A). HDACs 1 and 7 have been identified in two distinct repressive complexes, Sin3 and NuRD (Jepsen and Rosenfeld, 2002). However, only NuRD is ever present on the pS2 promoter in E_2-treated MCF-7 cells, as assessed by the Re-ChIP analysis illustrated in Figure 5B. HDACs 1 and 7 also associate on the pS2 promoter with SWI/

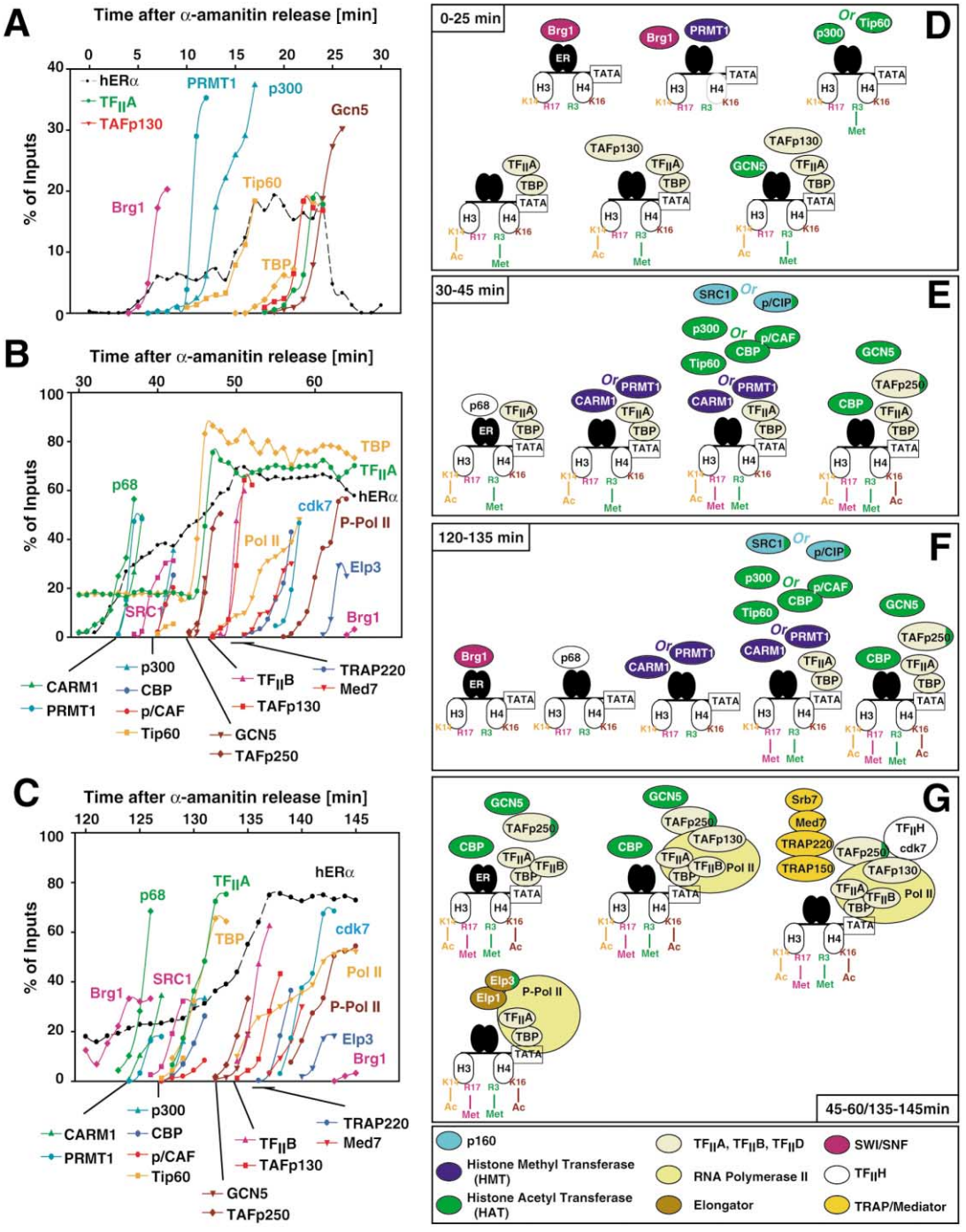

Figure 4. Events Directed by Liganded hERα on the pS2 Promoter that Results in Activation of Pol II

(A, B, and C) Kinetic ChIP experiments with 1 minute scale sampling. All ChIPs were performed from a single chromatin preparation at each time point. Results are expressed as in Figure 2.

(D to G) Depiction of protein engagement that occurs on the pS2 promoter, during the first transcriptionally unproductive cycle (D), at the commencement of the following cycles (E and F), and the protein recruitments common to the cycles illustrated in C and E (G).

SNF complexes, but NuRD components are not found with SWI/SNF complexes (Figure 5B). This suggests that HDACs not included in Sin3 or NuRD associate with SWI/SNF. Heat shock proteins (hsp) are central to the cycling of the glucocorticoid receptor on promoters (Freeman and Yamamoto, 2002). Interestingly, hsp70 but not hsp90 was found associated with NuRD (Figure 5B). Kinetically, when hsp70, HDACs and SWI/SNF are present on the pS2 promoter with maximum occupation, as the pS2 promoter is becoming refractory to hERα, NuRD (exemplified by its Mi2 component) is recruited only on the departure of TBP/TFIIA (Figure 5C). A subset

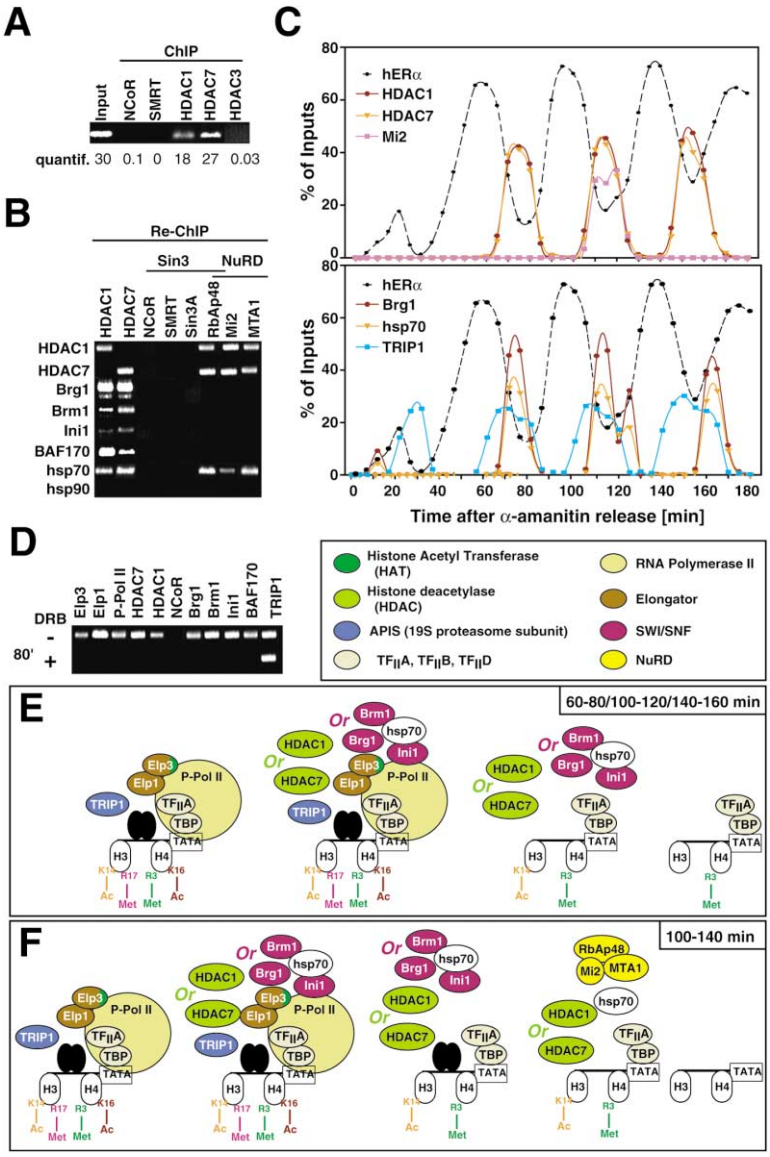

Figure 5. Recruitment of "Repressive Complexes," hsp and Proteasome Subunits on Active pS2 Promoter

(A) ChIP assays performed on chromatin prepared from cells treated with 10 nM Estradiol (E_2) for 3 hr.

(B) Re-ChIP analysis: Chromatin prepared from cells treated for 3 hr with 10 nM E_2 was subject to ChIP with antibodies shown at the left side, and then purified complexes were again subject to ChIP using antibodies shown at the top of the image.

(C) Kinetic ChIP experiments on chromatin prepared from cells after α-amanitin treatment and following 10 nM E_2 treatment. Results are expressed as in Figure 2.

(D) ChIP assays performed on chromatin prepared from α-amanitin synchronized cells after 80 minutes of 10 nM E_2 or E_2 + DRB treatment.

(E and F) Summary of events that achieve clearance of the pS2 promoter.

of the 20S proteasome, termed APIS, is involved in the "clearance" of hERα from the pS2 promoter (Reid et al., 2003), and we show here that APIS engagement precedes the recruitment of HDAC complexes, as revealed by the kinetics of recruitment of the TRIP1 component of APIS (Figure 5C). Inhibition of Pol II phosphorylation using DRB inhibits "clearance" of hERα from the pS2 promoter (Shang et al., 2000; Supplemental Figure S5 available on *Cell* website). Confirming the key role of this event, recruitment of all "clearing complexes" except APIS to the pS2 promoter is blocked when cells are treated by DRB (Figure 5D). Recruitment of APIS is thus a mechanistically separate event targeting liganded hERα for proteasome-mediated degradation (Reid et al., 2003). The conclusion of these experiments is summarized in Figures 5E and 5F.

Cyclic Remodeling of pS2 Promoter Nucleosomal Organization

We next considered the consequences of SWI/SNF and NuRD recruitment on the epigenetic status of the pS2 promoter, as both complexes exhibit chromatin-remodeling activities (Dillon and Festenstein, 2002; Jepsen and Rosenfeld, 2002). The pS2 promoter contains two phased nucleosomes, termed NucE and NucT, as they include at their edge either the ERE or the TATA box, respectively (Sewack and Hansen, 1997; Figure 6A). PCR performed on mononucleosome preparations (Figure 6B) show that the previously described immobile nucleosomes, NucE and NucT (Sewack and Hansen, 1997), are rather two preferred positions (Figure 6C). Indeed, 20 bp adjacent of the previously described positions can be protected from MNAse (micrococcal nuclease) digestion (as revealed by the amplification of the *F*, *G*, *I*, and *J* products after α-amanitin treatment in Figure 6C), a process that immobile nucleosomes would not allow. Furthermore, these preferred positions cyclically fluctuate, as exemplified by the regular variations in the amplifications of the *A* and *C* PCR products, and by the similarity of the PCR patterns obtained at 0 and 120 min. Importantly, the TATA box region is accessible to MNAse (compare the *C*, *I*, and *J* products in Figure

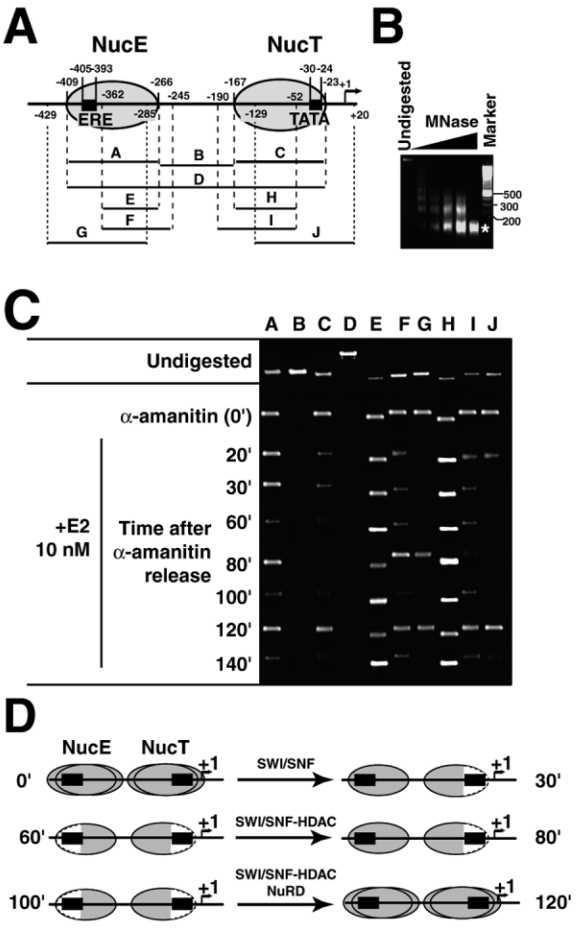

Figure 6. Dynamic Nucleosomal Positioning on the pS2 Promoter

(A) Location of nucleosomes of the pS2 promoter, organized around the ERE (NucE) and the TATA box (NucT), as identified by Sewack and Hansen (1997). Primer pairs used to amplify specific pS2 promoter regions are indicated.

(B) 5×10^5 MCF-7 cells were subject to DNA digestion with increasing amounts (0 to 10 U) of micrococcal nuclease (MNase). The asterisk points to the mononucleosome band.

(C) Mononucleosomes were prepared from MCF-7 cells synchronized by α-amanitin treatment, and treated with 10 nM estradiol (E_2). The nucleosome protected DNA fragments were then amplified by PCR using the *A* to *J* primer pairs illustrated. Absence of amplification with the *B* and *D* primer pairs determined that MNase digestion had occurred.

(D) Scheme summarizing dynamic histone modification and nucleosomal position within the pS2 promoter during hERα induced cycles. Dashed lines in the nucleosomes reflect the increased accessibility of the TATA and ERE surrounding regions for digestion by MNase.

6C), when TBP is loaded on the promoter (30–100 min after α-amanitin release, see Figure 4). This partial digest however does not reflect a phasing or moving of NucT, as the *I* or *J* products that would reveal a 5′or 3′ shift of the nucleosome, are not stabilized. Increased accessibility of the ERE sequence within NucE is also observed when the pS2 promoter is the more available for hERα binding, but not during the first transcriptionally unproductive cycle (20 and 30 min profiles, compare the *A*, *E*, *F*, and *H* products in Figure 6C). Four conclusions, which are illustrated in Figure 6D, can be drawn from these observations: (1) stabilization of both nucleosomes positions occur when SWI/SNF engages on the pS2 promoter after 0 and 120 min; (2) SWI/SNF induces an apparent disruption of DNA from NucT; (3) a cyclical access to the ERE region occurs, which is regulated by the SWI/SNF-HDAC complex during the clearance phase of each transcriptionally productive cycle; and (4) NuRD action appears to close NucT structure during the end of the first type of transcriptionally productive cycle.

Discussion

The aim of this study was to use ChIP based assays to determine the sequence of events resulting in the induction of the pS2 gene expression by hERα in MCF7 cells. A specific aspect of this report is the use of α-amanitin synchronized populations of cells, required for a preliminary silencing of the pS2 promoter.

Cycling of pS2 Promoter Transcriptional Competency: Roles for "Silencing Complexes"

Recurring fluctuations in core histone methylation and acetylation status characterize a cyclical permissiveness of the pS2 promoter for transcription and illustrate the concept of the histone code (Jenuwein and Allis, 2001). Indeed, acetylation of H3 K_{14} and dimethylation of H4 R_3 are permissive and define transcriptional competence of the pS2 promoter, as these modifications occur during the first transcriptionally nonproductive cycle. Analogously, posttranslational modifications of core histones and chromatin decondensation was observed in living cells in the absence of transcriptional initiation with the acidic VP16 activation domain (Tumbar et al., 1999). The additional dimethylation of H3 R_{17} and the acetylation of H4 K_{16} define a transcriptionally engaged pS2 promoter. In agreement with Daujat et al. (2002), our data indicate that H3 K_{14} acetylation occurs before dimethylation of H3 R_{17}, but kinetic resolution by ChIP demonstrates that these events do not occur within the same cycle. In contrast, dimethylation of H4 R_3 occurs before acetylation of H4 K_{16}. To be complete this analysis will now require the still unavailable antibodies specific for mono- or trimethylated residues of H3 and H4. Indeed, the potential different kinetics of these successive methylation events might generate a more complex histone code marking competent or transcriptionally engaged promoters (Jenuwein and Allis, 2001). However, H3 in the proximal promoter of the HNF4α gene is dimethylated prior to a wave of acetylation events that spreads from the enhancer to the TATA box (Hatzis and Talianidis, 2002). A different sequence was described for the collagenase promoter (Martens et al, 2003). It follows that the exact sequence of histone modification required for promoter commitment apparently depends upon the histone and upon the promoter.

Highlighting the key role of nucleosome remodeling in commitment of the pS2 promoter, following association of liganded hERα, SWI/SNF are the first complexes to be engaged on the promoter where they generate a stable nucleosome conformation permissive for transcription initiation (Figure 6). Conversely, a SWI/SNF-HDAC complex, similar to that described by Sif et al. (2001), is recruited after phosphorylation of Pol II, when

histone deacetylation commences. This confirms the role that Pol II has in the production and modulation of chromatin condensation (Muller et al., 2001) and provides an explanation for the observation that Pol II retains SWI/SNF on a promoter (Sharma et al., 2003). The occurrence of hsp70 (Figure 6) at the end of cycles induced by liganded hERα extends the proposed action of hsps in mechanisms of transcriptional regulation, in addition to their requirement in the clearance of glucocorticoid receptor from promoters (Freeman and Yamamoto, 2002). Indeed, hsp70 was found during the clearance phase of the pS2 promoter, in conjunction with SWI/SNF. Importantly, the NuRD complex, containing HDACs and remodeling activities, becomes recruited when TBP and $TF_{II}A$ depart from the promoter, and is correlated with the generation of a silenced chromatin context within the pS2 promoter (Figures 5 and 6). In summary, transcription initiation is achieved and controlled through opposing cyclical epigenetic processes that initiate and terminate with remodeling of chromatin.

Combinatorial Recruitment: Reflecting Functional Redundancy?

The multiple interactions between nuclear receptors and HATs, HMTs, and p160 families of cofactors gave rise to the hypothesis that alternate/combinatorial utilization of transcription factors is required for transcriptional activation (McKenna and O'Malley, 2002). Our Re-ChIP results demonstrate that such functional redundancies exist in vivo. For instance, p300 or CBP are combinatorially recruited to the promoter throughout the different cycles. Importantly, we found that combinatorial recruitment is a widespread feature of almost all steps leading to transcriptional activation and restriction, affecting not only the recruitment of HATs, HMTs, and p160, but also HDACs and ATPases from SWI/SNF complexes.

Systematic evaluation through Re-ChIP also helps identification of the molecular process required at precise steps in the cycles we describe. For instance, the alternative binding of either p/CAF or Tip60, a component of a multisubunit complex related to yeast NuA4 (Brown et al., 2001), achieves the same progress toward activation of the pS2 gene. Mechanistically, this suggests that only the HAT activity of the Tip60 complex is required at this step and not the ATPase or DNA helicase functions possessed by this protein (Ikura et al., 2000). However, such combinatorial recruitments also have limitations. For example, Brg1, but not Brm1-based SWI/SNF complex is exclusively found when TBP and $TF_{II}A$ are recruited to the pS2 promoter. As hERα contains two zinc fingers and Brg1 specifically interacts with zinc finger proteins (Kadam and Emerson, 2003), a physical interaction between Brg1 and hERα may be essential at this step. Importantly, p300, but not CBP, is present on the pS2 promoter during the initial unproductive cycle directed by liganded hERα (Figure 4), reflecting in vitro studies that found a key role for p300 in transcription initiation (Kraus and Kadonaga, 1998) and nonredundant roles between p300 and CBP (Schiltz et al., 1999). Beyond such functional redundancy is also the notion of recruitment preference, recently illustrated in the case of the engagement of p160 and HATs on promoters mediated by the glucocorticoid and progesterone receptors (Li et al., 2003). The systematic use of RNAi, in conjunction with ChIP assays, will determine the relative specificity and redundancy of the recruitment of all these factors.

Transcription Initiation: Stochastic but Sequential Events Controlled by an Allosteric "Transcription Clock"

An apparent discrepancy remains between the duration of cycles determined by ChIP and estimates of short residency times of transcription factors on promoters determined in live cell imaging experiments (McNally et al., 2000). However, both techniques evaluate different parameters. ChIP determines, at the time of assay, the proportion of target DNA sequence associated with a target protein, and not its persistence. Once a given protein binds to a promoter, it may rapidly dissociate and then bind again unproductively until stochastically an event occurs that achieves progress to the next step in the transcription cycle. Correspondingly, we describe "waves" of transcription and report quantitative data on the time required for transition from one step to another. In this regard, kinetics of P-Pol II association with the pS2 promoter do not indicate that transcriptional initiation occurs every 40 min, a conclusion that might have been contrary to earlier results (Kimura et al., 2002). Rather, such kinetics demonstrate that 5 to 10 min are required for Pol II to become activated (Figure 5). Underlying these concepts of stochastic and combinatorial events is the presence of a clock that synchronizes sequential waves of promoter accessibility for given cofactors and that acts to regulate progress through an ordered sequence of events to achieve transcriptional initiation.

The existence of this clock is illustrated by the occurrence of three different cycles (Figure 7). An initial transcriptionally unproductive process is required for promoter commitment, promoted through the engagement of SWI/SNF, which remodels nucleosomal organization of the pS2 promoter. The recruitment of HMTs, HATs, and then components of the basal transcription machinery follow this step. Following the action of GCN5 and TAF130, hERα becomes targeted to the proteasome by the APIS complex. This initial cycle is then followed by a transcriptionally productive cycle, where p68 RNA helicase is first recruited. Then, the combinatorial sequestering of HMTs, followed by p160 and HATs, ultimately form a large, previously uncharacterized complex on the promoter that directs and achieves further posttranslational modification of histones. The engagement of p160 prior to other HATs confirms the essential scaffold role of SRC1 and p/CIP in the construction of complexes involved in NR-mediated transactivation (Liu et al., 2001; Shang et al., 2000). The subsequent association of TAF250, TAF130 and TRAP/mediator emphasizes the role that TAF130 has in the recruitment of mediator (Guermah et al., 2001). Furthermore, mediator promotes the phosphorylation of Pol II through the cdk7 subunit of $TF_{II}H$. We observed that this Pol II phosphorylation event induces exchange of elongator onto the pS2 promoter. Given that elongator may have a limited role in transcription elongation in yeast (Pokholok et al., 2002), we do not exclude that an alternate complex may be

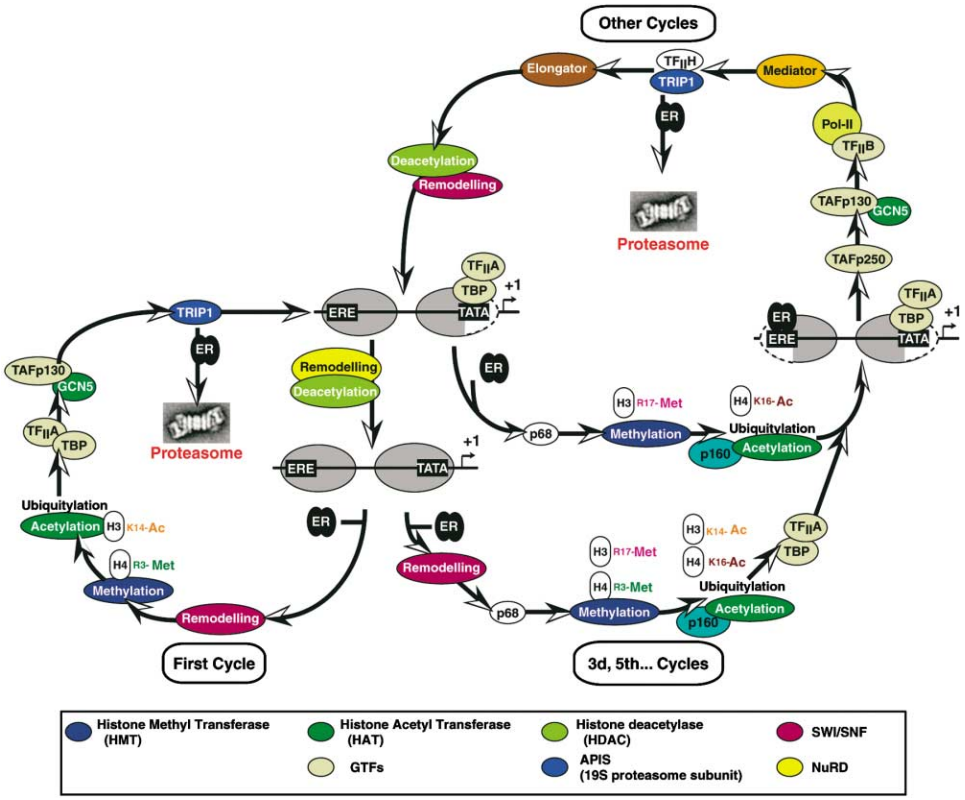

Figure 7. Model

This scheme summarizes our results reported here and in our previous report (Reid et al., 2003) for the three different, but linked, transcriptional cycles we determined. The left side shows events directed by E_2-bound hERα during the initial nonproductive cycle. The right image illustrates the two alternating productive cycles. For full description, see the Discussion section.

recruited at this stage. Following transcription initiation, hERα is again targeted to the proteasome by the APIS complex, and HDACs-SWI/SNF complexes remodel the nucleosomal organization of the pS2 promoter, allowing subsequent cycles to proceed. At the end of the second productive cycle, NuRD is specifically recruited to the promoter and presumably displaces remaining $TF_{II}A$/TBP complexes located on the TATA box of the promoter. A reinitiation cycle is then needed, where complete remodification of the nucleosomal structure of the pS2 promoter occurs prior to the commencement of subsequent cycles.

The step-to-step transitions between alternative cycles are likely to rely on the interplay between enzymatic activities of transcription factors and the epigenetic status of the promoter, allowing, for instance, the recruitment of NuRD only at the end of one productive cycle but not the end of the following cycle. Changes in the methylation/acetylation status of histones and nucleosome remodeling may record and direct progress through each cycle. For example, binding of CBP to promoters is alleviated by H3 methylation (Wang et al., 2001a), and acetylation of H4 stabilizes recruitment of SWI/SNF (Hassan et al., 2001). Moreover, allosteric changes induced within partner proteins during their physical interaction may also form signals for cycle progression, as suggested for CBP, p/CAF, and p160 (Perissi et al., 1999; Demarest et al., 2002) or for hERα following its interaction with TBP (Warnmark et al., 2001). Many HATs or HMTs modify target proteins other than histones (Wang et al., 2001b; Xu et al., 2001). Consequently, these posttranslational modifications may also constitute structural signals that induce sequential recruitment of different protein complexes, as seen with CBP and p160 (Chen et al., 1999). Lastly, protein ubiquitination may also be another regulatory event, as E3 ubiquitin ligases are recruited to the pS2 promoter at the HAT/HMT step (Reid et al., 2003 and data not shown).

A General Model for Transcription Regulation?

The model we detail in Figure 7 describes features likely to be general to transcriptional activation. For instance, when committed, the pS2 promoter has methylated and acetylated histones and has recruited TBP and $TF_{II}A$, as has been described for the HNF4α gene (Hatzis and Talianidis, 2002). However, the sequence of protein recruitment differs in other cell and promoter situations. For example, histone acetylation events precede SWI/SNF action on the IFNβ promoter (Agalioti et al., 2002). Further, many physical interactions that have been demonstrated between proteins that we analyzed do not result in their simultaneous presence on the pS2 promoter (compare Supplemental Table S2 available on *Cell* website and Figure 3). It follows, therefore, that there are subtle variations in steps that lead to the activation of transcription of different genes. Reconciling transcrip-

tion with cell organization, a main factor in these alternative mechanisms might rely on both genetic and spatial information given by the DNA sequence and its localization into chromatin subdomains, as discussed by Alvarez et al. (2003). This notwithstanding, the sequential process detailed in this study where 46 different proteins were monitored provides a solid basis for comparative analysis and outlines general features required for the achievement of transcription initiation. Specifically, and as suggested by Vermeulen and Houtsmuller (2002), our results depict transcription initiation resulting from the sequential and stochastic recruitment of specific protein complexes from a large panel of potentially redundant factors. In addition, transcription initiation generally may be cyclical, with cyclicity achieved through several possible mechanisms, including the action of APIS that targets transcription factors to proteasome-mediated degradation (Ferdous et al., 2002; Kang et al., 2002; Reid et al., 2003) and the roles we report for HDACs and remodeling complexes. Such waves of transcription authorization allow the cell to continuously adapt the transcription rate of a gene in response to differing situations through restricting the duration of activation. These processes are integral in the translation of hormone levels or any other signal into appropriate transcriptional responses.

Experimental Procedures

Reagents and Antibodies

α-amanitin, 5,6-dichlorobenzimadazole riboside (DRB), micrococcal nuclease (MNase), and estradiol (E_2) were purchased from Sigma (Taufkirchen, Germany). Antibodies were either gifts, or purchased from Advanced Immunochemicals (Freiburg, Germany), Affiniti Research (Exeter, United Kingdom), Santa Cruz (Heidelberg, Germany), or Upstate Biotechnology (Buckingham, England) (see Supplemental Table S6 available on *Cell* website). The functionality of these antibodies in our assays is illustrated in the Supplemental Figure S7 available on *Cell* website. Horseradish peroxidase-conjugated antibodies were from DiaNova (Saxvägen, Sweden).

Chromatin Immunoprecipitations (ChIPs), Re-ChIPs, and Immunoprecipitations (IPs)

Cells were maintained in DMEM (Sigma) supplemented with 5% fetal calf serum (FCS, Sigma) at 37°C under 5% CO_2. ChIP were performed with modifications of the procedure described by Shang et al. (2000), using 2×10^6 cells synchronized by 3 days of culture in by DMEM/2% dextran-charcoal treated FCS cells (blocking in G1/G2 phase; Villalobos et al., 1995) and then treated with 2.5 μM α-amanitin for 2 hr, followed by exposure to 10^{-8} M E_2 or ethanol including or not 25 μg/ml DRB. Chromatin was crosslinked using 1.5% formaldehyde for 5 min at 37°C, and the cells were collected after 2 washings with PBS in 1 ml cell collection buffer (100 mM Tris-HCl [pH 9.4] and 100 mM DTT). Cell suspension was then incubated on ice for 15 min and subsequently at 30°C for 15 min. Cells were then lysed sequentially by vortexing and 5 min centrifugation at 3000 $\times$ g at 4°C with 1 ml Buffer A (10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5] and 0.25%Triton X-100) and 1 ml Buffer B (1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5] and 200 mM NaCl), and sonicated 2 times for 15 s at maximum settings (Sonifier Cell Disruptor, Branson, Danbury, CT) in 100 μl lysis buffer [10 mM EDTA, 50 mM Tris-HCl [pH 8.0], 1% SDS, 0.5% Empigen BB (Sigma)]. Significantly, as we intended to consider the maximum of promoter molecules possible, we did not fragment chromatin under the range of 2–0.5 kb, as it is usually done (Kuo and Allis. 1999). After centrifugation, 15 μl of the supernatants were used as inputs, and the remainder diluted 2.5-fold in IP buffer (2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl [pH 8.1], and 0.5%Triton X-100). This diluted fraction was subjected to immunoprecipitation overnight after 2 hr preclearing at 4°C with 20 μl preimmune IgG (Sigma), 0.05% BSA, 5 μg sheared salmon sperm DNA, and 50 μl of a 50% protein A-Sepharose beads (Sigma) slurry. These beads were prepared by three washings in PBS, and resuspension in 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]. Complexes were recovered by a 2 hr incubation at 4°C with 2 μg of sheared salmon sperm DNA and 50 μl of protein A-Sepharose. Precipitates were serially washed with 300 μl Washing Buffer I (2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 0.1% SDS, 1% Triton X-100, 150 mM NaCl), Washing Buffer II (2 mM EDTA, 20 mM Tris-HCl [pH 8.0], detergents and NaCl in conditions determined for each antibody, as indicated in the Supplemental Table S6 available on *Cell* website), Washing Buffer III (1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 1% NP-40, 1% Deoxycholate, 0.25 M LiCl) and then twice with 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]. Precipitated chromatin complexes were removed from the beads through a 30 min incubation with 50 μl of 1% SDS, 0.1 M $NaHCO_3$, with vortexing each 5 min. This step was repeated twice, with 10 min incubation times. In Re-ChIP experiments, complexes were eluted by incubation for 30 min at 37°C in 25 μl 10 mM DTT. After centrifugation, the supernatant was diluted 20 times with Re-ChIP buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, [pH 8.1]) and subjected again to the ChIP procedure. All buffers contained 1$\times$ protease inhibitors cocktail (Complete minus EDTA, Roche). Crosslinking was reversed by an overnight incubation at 65°C. DNA was purified with QIAquick columns (Qiagen, Hilden, Germany), as indicated by the manufacturer, except that the samples were first mixed with agitation for 30 min with PB buffer. For the coimmunoprecipitations, 5×10^7 cells were lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS in PBS) followed by sonication twice for 15 s. Retained complexes were washed as described above. After immunoprecipitation as in the ChIP procedure, proteins were transferred onto PVDF membrane (Amersham Pharmacia, Piscataway, NJ, USA), and blots were developed with the ECL kit (Amersham Pharmacia).

Micrococcal Nuclease Digestion-Driven Preparation of Mononucleosomes

Mononucleosomes were prepared from 5×10^5 cells by MNase digestion following a method derived from the one described by Soutouglou and Talianidis (2002). After formaldehyde crosslinking and two washings with PBS, 5×10^5 cells were suspended in 1 ml nuclei buffer containing 25 mM HEPES, [pH 7.8], 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, 1 mM DTT, and protease inhibitor cocktail (Roche). Nuclei were then obtained by Dounce homogenization (4 strokes, pestle A) and sedimented by 20 min centrifugation at 4°C at 1400 $\times$ g through 1 ml 10 mM Tris-HCl [pH 7.4], 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, and 0.5 mM spermidine and 10% sucrose. The nuclear pellet was then resuspended in 100 μl digestion buffer (50 mM Tris-HCl [pH 7.5], 15 mM NaCl, 5 mM KCl, 3 mM MgCl2, 1 mM CaCl2, 10 mM $NaHS0_4$, 0.25 M sucrose, 0.15 mM spermine, 0.5 mM spermidine, and 0.15 mM mercaptoethanol) containing increasing concentrations of micrococcal nuclease (0 to 10 U; Sigma) for 30 min at 37°C. Reactions were stopped by the addition of 100 μl of stop solution (200 mM EDTA and 200 mM EGTA [pH 7.5]). Digested DNA was recovered by QIAquick columns (Qiagen) and 2 μl of the samples were submitted to PCR analysis. 10 μl were also migrated in Ethidium Bromide stained gel to control the efficiency of the digestion.

RT-PCRs and PCRs

RNA were prepared from sampled cells by the TriZol reagent (Invitrogen, Carlsbad, CA). Reverse-transcription using poly-dT oligos (Roche) was then performed on 1 μg RNA treated with DNAse (Roche). The 5′ to 3′ sequences of the primers used in the PCR (synthesized by MWG GmbH, Ebersberg, Germany) are: pS2 qPCR Fwd: ATGGCCACCATGGAGAACAA; pS2 qPCR Rev: TAAAACAGTG GCTCCTGGCG; GAPDH qPCR Fwd: TCTGGTAAAGTGGATATTG TTG; GAPDH qPCR Rev: GATGGTGATGGGATTTCC; −701Fwd: TGA TTCTCCTGACTTAACCTCC; −517Rev: CACGCTGTAATCCCAACA CTTTG; −350Fwd: GTTGTCAGGCCAAGCCTTTT; −30Rev: GAGCG TTAGATAACATTTGCC; −430Fwd: ATTAGCTTAGGCCTAGAC; −409Fwd: ATGGGCTTCATGAGCTCC; −362Fwd: CCACTGTTGT CAGGCCAAGC; −285Rev: CTGCAGAAGTGATTCATA; −266Rev:

AGGGTAAATACTGTACTCAC; −245Rev: TACTCATATCTGAGAGGC CCT; −266Fwd (Linker): CTGGCGGGAGGGCCTCTCAGA, −167Rev (Linker): CTGAGGGATCTGAGATTCA; −190Fwd: GAAAGAGGGACT TTCTGAATC; −167Fwd: CAAGATGACCTCACCACATG; −129Fwd: TCTGTCTATCAGCAAAT; −52Rev: GCGTTAGATAACATT; −23Rev: GGATTTTATAGGGCAG; and +20Rev: TTGCCTCCTCTCTGCTCC AAA. Quantitative PCR were performed using SybrGreen (Molecular Probes, Eugene, Oregon) as marker for DNA amplification on a SmartCycler (Eurogentec, Seraing, Belgium) or an ABI Prism 7000 apparatus (Applied Biosystems, Foster City, CA), with 60 cycles of three-step amplification. Detectable amplification of specific products arose between 13 to 28 cycles.

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