Transcriptional complexes engaged by apo-estrogen receptor- α isoforms have divergent outcomes

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Unliganded (apo-) estrogen receptor α (ER α , NR3A1) is classically considered as transcriptionally unproductive. Reassessing this paradigm demonstrated that apo-human ER α (ER α 66) and its N-terminally truncated isoform (ER α 46) are both predominantly nuclear transcription factors that cycle on the endogenous estrogen-responsive pS2 gene promoter *in vivo*. Importantly, isoform-specific consequences occur in terms of poisoning the promoter for transcription, as evaluated by determining (i) the engagement of several cofactors and the resulting nucleosomal organization; and (ii) the CpG methylation state of the pS2 promoter. Although transcriptionally unproductive, cycling of apo-ER α 66 prepares the promoter to respond to ligand, through sequentially targeting chromatin remodeling complexes and general transcription factors. Additionally, apo-ER α 46 recruits corepressors, following engagement of cofactors identical to those recruited by apo-ER α 66. Together, these data describe differential activities of ER α isoforms. Furthermore, they depict the maintenance of a promoter in a repressed state as a cyclical process that is intrinsically dependent on initial poisoning of the promoter.

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Introduction

Estrogens, such as 17- β estradiol (E₂), are steroid hormones predominantly synthesized by ovaries that act as pivotal

hormones in the physiology of the female reproductive tract and in the homeostasis of other tissues (Nilsson *et al*, 2001). Although protective against pathologies such as osteoporosis, Alzheimer's and cardiovascular disease, E₂ has roles in the ontogeny of breast cancer (Bian *et al*, 2001; Jordan *et al*, 2001; Honjo *et al*, 2003). Most functions exerted by E₂ are mediated by estrogen receptors (ER α and ER β ; NR3A1 and NR3A2; NR Committee, 1999) that modify, through binding ERs to their promoter region, the pattern of expression of specific target genes. This can occur either directly through interaction with cognate DNA sequences (EREs or estrogen-responsive elements) or through protein/protein interaction with other transcription factors.

ERs belong to the nuclear receptor (NR) superfamily of transcription factors (Robinson-Rechavi *et al*, 2003), whose sequence can be divided into six domains (A–F). The central C region is responsible for DNA sequence recognition and cooperative binding of ER dimers on EREs that contain a palindromic PuGGTCA motif separated by 3 bp (Krust *et al*, 1986). E₂ lodges in a hydrophobic pocket defined by the C-terminal E domain (ligand binding domain (LBD)). This LBD also contains a dimerization interface that stabilizes receptor dimers. Binding of E₂ induces a considerable three-dimensional remodeling of ERs, generating surfaces that can associate with intermediate transcription factors. These surfaces are included within the two transactivation functions (AFs) of ERs, located in the N- (AF1) and C-terminal (AF2) domains (Brzozowski *et al*, 1997; Robinson-Rechavi *et al*, 2003). ER α induces the ordered recruitment of a series of coactivator complexes, leading to histone acetylation, methylation, chromatin remodeling and recruitment of the basal transcription machinery (Shang *et al*, 2000; Belandia and Parker, 2003; Métivier *et al*, 2003). To date, six classes of coactivator complexes have been identified as interacting with ER α in a ligand-dependent manner: (i) members of the p160 subfamily (exemplified by SRC1 and TIF2); (ii) histone acetyl transferases (HATs) such as CBP/p300; (iii) histone arginine methyl transferases (HMTs) such as CARM1 or PRMT1; (iv) nucleosome remodeling complexes such as SWI/SNF; (v) complexes of the SMCC/TRAP/DRIP/ARC class that facilitate the activation of the polymerase II (PolII); and (vi) the AF1-specific coactivators p68 and p72 RNA helicase (Robyr *et al*, 2000; Watanabe *et al*, 2001). In contrast, ER α liganded to antiestrogens such as 4-hydroxytamoxifen (OHT) downregulates transcription. The three-dimensional changes provoked by binding of OHT induce recruitment of corepressors such as NR corepressor (NCoR) and histone deacetylase (HDAC) (Jepsen and Rosenfeld, 2002).

The MCF7 human breast cancer cell line expresses, in addition to ER α 66, an isoform 46 kDa in size (ER α 46), encoded by an RNA variant produced by alternative splicing (Flouriot *et al*, 2000). Expression of ER α 46 was also reported

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in osteoblasts (Denger *et al*, 2001) and in endothelial cells, in which this protein exhibits partial membrane localization (Li *et al*, 2003). Functionally, ER α 46 lacks the amino-terminal A/B domain present in the full-length ER α 66, and consequently is devoid of AF1. Also, ER α 46 suppresses the AF1 activity of ER α 66 (Flourit *et al*, 2000). Finally, unliganded (apo-) ER α 46 may repress transcription of target genes (Mtvier *et al*, 2002). We recently described the sequence of protein recruitment engaged by ER α 66 in MCF-7 cells in the presence of E₂ that is required to activate the endogenous E₂-responsive pS2 gene (Mtvier *et al*, 2003). As both isoforms exhibit distinct activities in the absence of hormone, we sought to describe the mechanisms occurring during the ligand-independent association of ER α 46 and ER α 66 isoforms with the pS2 promoter.

Results

Apo-ER α isoforms are predominantly nuclear proteins and differently impact the expression of the pS2 gene

Concomitant expression of ER α 46 and ER α 66 in MCF7 cells prevented elucidation of their individual modes of action. We therefore stably expressed each isoform in ER α -negative MDA-MB231 cells, giving rise to MDA::ER α 66 and MDA::ER α 46 cells. Immunostaining using HC20, which targets both isoforms (Figure 1A), shows that ER α 46 and ER α 66 are similarly distributed in these cells, residing mainly in the

nucleus (Figure 1B). Functionally, expression of ER α 66 or ER α 46 in MDA-MB231 cells induces ligand-dependent expression of the endogenous estrogen-inducible pS2 gene similar to that observed in MCF7 cells (Figure 1C). However, in the MDA::ER α 46 cell line, the level of pS2 mRNA was three-fold lower in the absence of E₂ than in MDA::ER α 66 cells (Figure 1C). Chromatin immunoprecipitation (ChIP) assays using unsynchronized cells demonstrate that the E₂-induced expression of the pS2 gene reflects binding of ER α and of activated/phosphorylated RNA PolII (P-PolII) to the promoter (Figure 1D). Furthermore, the pS2 promoter contains acetylated histones (Ac-Hist), a marker of an active transcription locus (Sternr and Berger, 2000), in cells only when they express ER α (Figure 1D). The relative amounts of P-PolII and Ac-Hist present on the pS2 promoter were significantly lower in MDA::ER α 46 cells than in ER α 66-expressing cells in the absence of E₂, suggesting that apo-ER α 46 specifically represses basal transcription of the pS2 gene.

Does the apo-ER α 46-mediated silencing of the pS2 promoter involve DNA methylation processes?

Long-term silencing of genes is often associated with an increased CpG methylation (Bird, 2002). To question the involvement of these epigenetic modifications in the actions of apo-ER α 46, we evaluated the methylation status of CpGs present within the pS2 promoter (Figure 2A). Digestion of

Figure 1 Functional properties of ER α isoforms. (A) Scheme illustrating ER α isoforms sequence and the location of the epitopes targeted by antibodies used. (B) Immunostaining of ER α isoforms with HC20. MCF-7 cells expressing both isoforms and MDA-MB231 cells expressing none or either isoforms (MDA::ER α 66 or MDA::ER α 46) were fixed after a 3 h treatment with 10⁻⁸ M estradiol (E₂) or ethanol (EtOH) as vehicle control. Nuclei are visualized by Hoechst staining. (C) The expression of endogenous pS2 gene in the four cell lines was monitored by real-time PCR. After 72 h of culture in stripped media, cells were treated for 3 h with 10⁻⁸ M E₂ or EtOH as vehicle control. pS2 mRNA levels were normalized against invariant GAPDH mRNA. (D) ChIP probed the recruitment of ER α , P-PolII and Ac-Hist to the pS2 promoter in unsynchronized cells sampled as in (C).

Figure 2 Evaluation of intercellular variations in ^{Me}CpG content of the pS2 promoter. **(A)** Schematic representation of the pS2 promoter, with significant *cis*-acting features and phased nucleosomes (Sewack and Hansen, 1997) highlighted. CG dinucleotides are indicated, with those within an *Hpa*II site in gray. Regions amplified by PCR primers are also depicted. **(B)** Genomic DNA prepared from MDA-MB231, MCF-7 and MDA-MB231 cells stably expressing either ER α isoforms was digested by *Hpa*II and then subject to PCR to amplify the described regions. **(C)** To quantify the methylation of given CpG, genomic DNA was chemically modified using bisulfite-mediated C to U transformation. The upper strand of the pS2 promoter was then specifically amplified by PCR, and the total pool of fragments was sequenced. Values are the mean $\pm$ s.e.m. from data acquired in three separate experiments.

genomic DNA isolated from the four cell lines by *Hpa*II (does not digest methylated or hemimethylated C^{Me}CGG) limited PCR amplification of the B, C and D fragments to varying degrees without disturbing amplification of the control A fragment (Figure 2B). While these three CpGs are likely fully methylated in MDA-MB231 cells, their methylation status is lower or even null in MCF7 and in MDA cells expressing either ER α isoform. These variations do not

correlate with different proportions of cells engaged in the S or G2 phases, which are points where replication-induced methylation occurs (Urnov, 2003; data not shown). Sequencing of PCR-amplified upper strand of the pS2 promoter, following bisulfite modification of genomic DNA, demonstrates that all 19 CpGs are methylated in MDA-MB231 cells, reflecting the silent state of the pS2 promoter in these cells. In contrast, CpGs located 3' to the -555 position exhibit a lower

Figure 3 Cyclical permissiveness of the pS2 promoter for ER α 46 and ER α 66 bindings, with distinct functional consequences. (A) Kinetic ChIP experiments performed using H184 or HC20 antibodies. Cells were first synchronized by a 72 h deprivation of serum and then treated for 2 h with 2.5 μ M α -amanitin. After washing and renewal of the media, chromatin was prepared on sampled cells with 5 min intervals. Amounts of immunoprecipitated pS2 promoter were quantified by real-time PCR. Values, expressed as the percent of inputs, are the mean of three separate experiments, and have an s.d. < 2%. (B) No differences in the cycling of both ER α isoforms on the pS2 promoter are evidenced in kinetic ChIP assays performed using chromatin prepared from MDA-MB231 cells expressing either ER α isoforms. Next, experiments probed the functional impact of these cycles of ER α isoforms binding in MDA::ER α 66 (C) and MDA::ER α 46 cells (D). These assays evaluated the recruitment and activation of P-PoIII in response to 10^{-8} M estradiol (E₂) added at times indicated within the graphs (A–C of each panels).

and similar methylation status in all cells expressing ER α isoforms (Figure 2C). Therefore, the methylation status of the pS2 promoter is not modified by the ER α 46 isoform. These results provide evidence that both ER α isoforms poise

the pS2 gene for expression and, overlying this effect, that apo-ER α 46 represses the basal transcription of the pS2 gene through mechanisms distinct from gene silencing by CpG methylation.

Cyclical binding of apo-ER α isoforms to the pS2 promoter has different effects

Association of liganded and apo-ER α 66 with target promoters is cyclical *in vivo* (Reid *et al*, 2003). We therefore evaluated the properties of the binding of apo-ER α 46 to DNA. Basal transcription of the pS2 gene in the absence of E₂ occurs in MCF-7 and stably transfected MDA cells, even after 3 days of steroid deprivation (Figure 1C). Transcription factors present on the promoter limit the clear analysis of the kinetics of apo-ER α binding. We abrogated this background activity of the pS2 promoter by treating cells for 2 h with 2.5 μ M α -amanitin, as described previously (Reid *et al*, 2003). Following the removal of α -amanitin, a maximum of 20% of endogenous pS2 promoters can be recovered by ChIP in the absence of E₂ using either the ER α 66-specific (H184) or HC20 antibodies in MCF7 cells (Figure 3A). Both antibodies gave similar curves with apo-ER α cycling with a periodicity of 20 min. The similarity of the curves obtained MDA::ER α 66 and in MDA::ER α 46 (Figure 3B) further demonstrates that both apo-ER α isoforms have similar cyclical DNA binding characteristics *in vivo*.

Unlike apo-ER α 66, apo-ER α 46 represses transcription (Mtvier *et al*, 2002), as illustrated by the lower expression of pS2 mRNA in MDA::ER α 46 cells in the absence of ligand (Figure 1C). We reasoned that differential poisoning of the pS2 promoter by the two apo-ER α isoforms may result in different dynamics in gene responses to E₂. After α -amanitin synchronization, cells were thus treated with 10 nM E₂ at different times and the activation of PolII on the pS2 promoter was followed. These experiments demonstrate that apo-ER α 66 initiates specific events sufficient for immediate response to E₂ (Figure 3C), bypassing, or equivalent, to those occurring during the first transcriptionally unproductive cycle observed in the presence of E₂ (Mtvier *et al*, 2003). In contrast, binding of apo-ER α 46 slows the response to E₂, as P-PolII binding is observed only if E₂ is added when ER α 46 has finished one of its cycles (Figure 3D). Therefore, while apo-ER α 66 poises the pS2 gene for expression, apo-ER α 46 inhibits pS2 gene commitment.

Recruitment of transcription factors by both ER α isoforms

MCF7 cells, which express both ER α 66 and ER α 46, were initially used to identify proteins recruited by both ER α isoforms to the pS2 promoter through comparison of ChIP patterns obtained on chromatin prepared from untreated MCF7 cells and from cells synchronized by α -amanitin. Without this synchronization step, ER α -independent initiating events occur, mediated by factors such as AP1 (binding site close to the ERE; Figure 2A), and are likely responsible

Figure 4 Identification of the transcription factors targeted by apo-ER α isoforms on the pS2 promoter *in vivo*. (A) Real-time PCR-mediated quantification of ChIP experiments performed using chromatin prepared from MCF-7 cells either treated with ethanol as vehicle control (EtOH) or firstly synchronized by α -amanitin treatment. Comparing the results obtained in both conditions enables determination of proteins directly recruited by both apo-ER α isoforms on the pS2 promoter (those after α -amanitin treatment, in gray). (B) Comparative ChIP assays using chromatin prepared from MCF-7, MDA-MB231 or MDA-MB231 cells expressing either ER α isoforms.

Figure 5 Re-ChIP screening of the factors recruited to the pS2 promoter by apo-ER α isoforms. Chromatin prepared from α -amanitin synchronized MDA-MB231 cells expressing ER α 66 (**A**) or ER α 46 (**B**) was subject to the ChIP procedure with the antibodies shown at the left side, and again immunoprecipitated using antibodies shown at the top of the panels. (**C**) Scheme illustrating the topology of the complexes recruited to the pS2 promoter, as indicated through the comprehensive Re-ChIP analysis shown above.

for the basal transcription of the pS2 gene (Barkhem *et al*, 2002; Supplementary Figure S1). Hence, α -amanitin-induced clearance of the pS2 promoter is essential to assess kinetically the recruitment of transcription factors to the promoter mediated by apo-ER α (Reid *et al*, 2003; Supplementary Figure S1). Subsequent ChIP assays demonstrate that, in MCF7 cells, either apo-ER α isoform or both have the ability to promote the engagement of HATs (Tip60, GCN5 and p300, but not CBP), HMT (only PRMT1), general transcription factors (only TBP and TFIIA) and repressive complexes (Sin3 and NuRD) on the pS2 promoter (Figure 4A, compare black and gray bars). As anticipated, transcription factors such as elongator or PolII, whose presence indicates an active pS2 gene, are not recruited by apo-ER α isoforms (Figure 4A). H3 was acetylated on K₁₄ but not dimethylated on R₁₇; H4 was dimethylated on R₃ but not acetylated on K₁₆. This is in contrast to an active pS2 promoter organization, where all these histone residues are modified (Mtievier *et al*, 2003).

Comparison of ChIP patterns obtained with MDA::ER α 66, MDA::ER α 46 and MCF7 cells confirms that both apo-ER α isoforms promote engagement of HATs and PRMT1 (Figure 4B). Importantly, although TBP engages the pS2 promoter in all cell lines, recruitment of TFIIA and TAFp130 occurs only in cells that express ER α 66. Conversely, components of the Sin3 repressive complex (NCoR/SMRT) are present on the pS2 promoter only in MDA::ER α 46 cells (Figure 4B). This is associated with lower acetylation of H3 K₁₄ as compared to MDA::ER α 66 cells. Recruitment of NuRD (Mi2 and MTA1) to the pS2 promoter may not be a repressive event but reflect a clearance process similar to that we described recently (Mtievier *et al*, 2003). Re-ChIP next elucidated the organization of transcription complexes recruited by both ER α isoforms to DNA (Figure 5A and B). Recruitment of HATs (p300, GCN5 and Tip60) and of HDACs are combinatorial, reflecting functional redundancy among these protein subfamilies. In summary, Re-ChIP identified five different protein complexes, of which two are ER α

Figure 6 Kinetics of the recruitment of transcription factors directed by apo-ER α isoforms on the pS2 promoter. **(A, B)** Kinetic ChIP experiments were performed and expressed as in Figure 4, using antibodies and chromatin prepared from synchronized MDA-MB231 cells expressing either ER α isoforms, as indicated within the graphs. **(C)** Scheme summarizing the sequence of recruitment of transcription factors directed by ER α 66 and ER α 46 onto the pS2 promoter. The numbering reflects the order of engagement of the different complexes onto the promoter. In the case of ER α 46, V-a and V-b are combinatorial steps.

isoform specific (Figure 5C): (i) complex IV containing GCN5 together with TBP, TF_{II}A and TAFp130 is specific for ER α 66; and (ii) the Sin3 repressive complex is specifically targeted by apo-ER α 46.

Kinetics of recruitment of transcription factors by apo-ER α isoforms

Following ER α and PRMT1 engagement on the pS2 promoter, Tip60 and p300 are recruited together (Figure 6A). Re-ChIP analysis shows that these HATs are present in different complexes (Figure 5), suggesting that both proteins are alternatively recruited to different sets of pS2 promoters. These data also confirm that PRMT1 methylates H4 R₃ (Wang *et al*, 2001). Of the 20 factors evaluated, only TAFp130, p300, GCN5 and Tip60 associate with the pS2 promoter with a frequency identical to ER α 66 (Figure 6Aa and b). In contrast, the pS2 promoter remains permissive for the association of TBP and TF_{II}A for about 1 h, with GCN5 association slightly increased when TBP and TF_{II}A load onto the promoter (Figure 6Aa and b). This suggests that GCN5 is required for the recruitment of TBP and TF_{II}A, or *vice versa*. ChIP analysis with a resolution of 1 min confirms these conclusions and further shows that PRMT1 is the first protein recruited to the pS2 promoter by either ER α isoforms. This is then followed by p300, Tip60 and GCN5 (Figure 6Ac). This analysis also shows that TBP is recruited 3–4 min before TF_{II}A. In MDA::ER α 46, the kinetics and sequence of association of a few of these proteins with the pS2 promoter were different, even when probed at 1 min intervals (data not shown). Notably, recruitment of TBP occurred on fewer pS2 promoters than in MDA::ER α 66 cells (Figure 6Ad and e). The most striking difference between the actions of apo-ER α isoforms is the specific recruitment of Sin3 repressors by apo-ER α 46 (Figure 6B). As summarized in Figure 6C, both apo-ER α isoforms recruit, with similar kinetics, transcriptional activators such as HATs and HMTs. However, following these steps, where the pS2 promoter becomes permissive for transcriptional activity, ER α 46 specifically recruits repressive complexes.

In the presence of E₂, cyclic clearance of the pS2 promoter of transcription factors is generated by (i) proteasome-mediated degradation of ER α through recruitment of the APIS complex (Reid *et al*, 2003); and (ii) recruitment of HDACs-SWI/SNF and NuRD complexes (Métiévier *et al*, 2003). Kinetic analysis of the recruitment of NuRD and TRIP1 (a component of the APIS complex) in MDA::ER α 66 cells indicates that NuRD may be involved in the removal of TF_{II}A and TBP from the pS2 promoter, while the APIS complex might cyclically remove ER α 66 from the pS2 promoter (Figure 6Ab, e and B). Unfortunately, cyclical recruit-

ment of NuRD and Sin3 that occurs in MDA::ER α 46 cells precludes a similar description of the action of these complexes in the generation of ER α 46 cycles. However, recruitment of TRIP1 at the end of the cycles driven by apo-ER α 46 (Figure 6Ae) indicates that, as for apo-ER α 66, APIS is key in clearing ER α from the pS2 promoter.

Apo-ER α induces changes in the nucleosomal organization of the pS2 promoter

We next verified the consequences of engagement of HATs, HMT and HDACs on chromatin organization within the pS2 promoter. In MDA::ER α 66 cells, histones H3 and H4 become, respectively, acetylated or methylated to constant levels following recruitment of p300 or Tip60 and PRMT1 to the pS2 promoter (Figures 6A, C and 7A). In contrast, in MDA::ER α 46 cells, acetylation of H3 K₁₄ parallels ER α 46 binding, with dimethylation of H4 R₃ exhibiting a more complex pattern. Repressors specifically recruited by apo-ER α 46 may thus impact on the acetylation levels of H3 K₁₄. In ER α -positive cells, ChIP assays performed on mononucleosomes generated by micrococcal nuclease (MNase) digestion of chromatin show both nucleosomes NucE and NucT (Sewack and Hansen, 1997; Figure 7B) to include dimethylated H4. In contrast, acetylation of H3 K₁₄ only occurs on NucT (Figure 7C). The position of NucE and NucT vary during cycles directed by ER α isoforms (Figure 7D). In ER α -positive cells, stabilization of both nucleosomes in the preferred positions described by Sewack and Hansen (1997) is first observed at 20 min (A and C). In both MDA::ER α 66 and MDA::ER α 46 cells, the region encompassing the TATA box becomes accessible to MNase (compare the C, H, I and J products in Figure 7D) when TBP associates with the promoter (20 min but not at 90 min; Figure 6A). At 90 min, the J fragment, which is digested in MDA::ER α 66 and MCF7 cells, is protected in MDA::ER α 46 cells. This indicates that NucT is more flexible within MDA::ER α 46 than in MDA::ER α 66 and MCF7 cells, where NucT protects only the 5' extremity, as revealed by amplification of the I fragment (Figure 7D). NucE also has dynamic flexibility in its position encompassing the 3' end of the sequence, as the F but not G fragment is protected from MNase in all cells. NucE adopts a fixed position in MCF7 and in MDA::ER α 66 cells at 30 and 90 min, when the pS2 promoter is free of ER α . In contrast, NucE has a higher flexibility in MDA::ER α 46 cells at these time points. Re-ChIP assays on mononucleosomes demonstrate that, at 20 min, dimethylated H4 R₃ and acetylated H3 K₁₄ are present together in the same pool of NucT (Figure 7E). It is only in MDA::ER α 66 that an additional dimethylation of H4 R₃ appears within NucE at 40 min (Figure 7E). Moreover, it is only in MDA::ER α 46 cells that levels of acetylated H3 K₁₄

Figure 7 Dynamic chromatin modifications within the pS2 promoter. (A) Kinetic ChIP experiments were performed as depicted in Figure 4, using antibodies and chromatin prepared from synchronized MDA-MB231 cells expressing either ER α isoforms, as indicated within the graphs. (B) Schematic representation of the pS2 promoter, with phased nucleosomes and regions amplified by PCR primers depicted. (C) Mononucleosomes prepared from indicated cells were used in ChIP assays detecting modified histones in both NucE and NucT. The absence of amplification using the B and D primer pairs controls proper digestion. (D) Fluctuations in NucE and NucT positions on the pS2 promoter were assessed through PCR amplification of mononucleosomes using the A to J primer pairs. (E) Following α -amanitin synchronization, mononucleosomes were prepared at times indicated and subjected to ChIP and Re-ChIP procedures using the indicated antibodies. Use of the E and H primer pairs for DNA amplification showed the localization of the modified histones within either NucE or NucT. (F) Scheme summarizing the dynamic histone modifications and nucleosomal organization of the pS2 promoter during ER α isoforms cycles. Dashed lines in NucT reflect the increased accessibility of the TATA box surrounding region for MNase digestion. The recruitment events that are hypothesized to correlate with the observed nucleosome modifications are boxed and highlighted.

in NucT cycle functionally reflect the sequential associations of HATs and HDACs (Figures 6 and 7D). Conclusions from these experiments are presented in Figure 7F.

Discussion

In addition to a predominant 66 kDa protein, the ER α gene also produces a 46 kDa isoform, lacking N-terminal domains, that has the ability to repress transcription in the absence of ligand (Flouriou *et al*, 2000; Métivier *et al*, 2002). This prompted us to determine the sequence of recruitment of transcription factors engaged by both apo-ER α isoforms *in vivo*. Cyclical engagements of apo-ER α isoforms at the pS2 promoter have a periodicity of 20 min, are transcriptionally unproductive and differ in their influence on pS2 promoter commitment.

Apo-ER α 66: achieving commitment of the pS2 promoter

The expression of ER α is necessary to determine pS2 gene activity (Figure 1). Correspondingly, if apo-ER α 66 is allowed to achieve one cycle of DNA binding prior to the addition of E₂, rapid (10 min) recruitment of PolII to the pS2 promoter occurs. This indicates that apo-ER α 66 induces chromatin modifications sufficient to permit subsequent transcription of the pS2 gene in the presence of liganded ER α 66. Induction of transcriptional competence by apo-ER α 66 involves the cyclical engagement of HATs and HMTs that acetylate and methylate H3 K₁₄ and H4 R₃, respectively. These events impact on the phased nucleosomal structure of the pS2 promoter, with the nucleosome associated with the TATA box (NucT) subject to two different events. First, NucT becomes stabilized into an 'active' position corresponding to that described by Sewack and Hansen (1997) followed by a disruption of DNA-histone contacts around the TATA box (Figure 7). SWI/SNF complexes stabilize the position of NucT in the presence of ligand (Métivier *et al*, 2003). As these complexes are not engaged on the pS2 promoter in the absence of E₂ (Figure 4), apo-ER α 66 may mobilize another remodeling complex, involved in the unfolding of large-scale chromatin structures described by Nye *et al* (2002). Stabilization of NucT in favored positions may also be an allosteric consequence of ER α binding, with this engagement stabilizing the overall equilibrium of NucT positions in the cell population towards the preferred positions. Second, dissymmetric flexibility of NucT occurs when TBP is cleared from the pS2 promoter, probably under the influence of NuRD. Recruitment of certain general transcription factors by apo-ER α 66 also accounts for the poisoning of the pS2 gene for transcription. As described for the HNF4 α gene (Hatzis and Talianidis, 2002), once prepared for transcription, the pS2 promoter has recruited TBP and TFIIA. Importantly, TAFp130 is targeted to the pS2 promoter with a similar frequency as ER α 66 but not TBP, which persists over successive cycles. In addition to its role in relieving the inhibition mediated by TAFp250 and in the recruitment of the mediator complex, TAFp130 may thus play additional roles (Guermah *et al*, 2001).

Apo-ER α 46: promoting repression of the pS2 promoter

A repressive role for apo-ER α 46 was suggested by the observation that, in the absence of E₂, the levels of pS2 mRNA were three-fold lower in MDA::ER α 46 cells than in

MDA::ER α 66 cells. ChIP assays demonstrated that apo-ER α 46 specifically recruits Sin3 and NuRD repressive complexes to the pS2 promoter. Although exhibiting a similar cycling frequency, the integration of the area covered by the respective binding curves of either apo-ER α isoforms indicates that around 20% more pS2 promoters are engaged in binding apo-ER α 46. Within a single cycle of pS2 association with apo-ER α , this difference indicates that, stochastically, binding of ER α 46 to DNA persists longer than ER α 66 does, reflecting the recruitment of additional repressive complexes. The Sin3 complex is recruited when ER α 66 is liganded with OHT (Jepsen and Rosenfeld, 2002). However, deletion of the entire N-terminal region of the receptor, as occurs in the ER α 46 isoform, prevents the A domain to hinder the interaction surface for corepressors in the absence of E₂ (Métivier *et al*, 2002). Additionally, this deletion abrogates the AF1 activity, generally accepted as a function that can be active or activated in the absence of E₂ (Shao and Lazar, 1999). The low basal pS2 gene activity observed in MDA::ER α 46 cells may therefore result from a combination of an active repressive process and a passive mechanism caused by the deletion of AF1. However, treating MDA::ER α 46 cells with ICI_{182,780}, an antagonist that inhibits ER α binding to DNA (Fawell *et al*, 1990), restored the basal mRNA level of pS2 observed in MCF7 and MDA::ER α 66 cells to 80% (data not shown). Correspondingly, the deletion of the AF1 domain may be responsible for 20% of the loss of basal pS2 expression seen in MDA::ER α 46 cells.

These active repressive actions of apo-ER α 46 do not involve stable long-term remodeling of chromatin exemplified by the inherited methylation of cytosines within the dinucleotide CpG (Bird, 2002). Instead, apo-ER α 46 induces unstable chromatin modifications through the combinatorial engagement of Sin3 and NuRD complexes onto the pS2 promoter that deacetylate K₁₄ of H3 of NucT and that achieve cyclic, asymmetric positioning of NucE. As with activating complexes (Métivier *et al*, 2003), alternative and combinatorial mobilization of proteins with a similar function governs stochastic mechanisms that affect transcriptional competence. Our Re-ChIP experiments confirm the existence *in vivo* and on DNA of biochemically defined alternative repressive complexes. Indeed, Sin3 complexes containing either NCoR or SMRT can recruit either HDAC1, 3 or 7 (Heinzel *et al*, 1997; Kao *et al*, 2000; Guenther *et al*, 2001). NuRD either contained HDAC1 or 7, but never HDAC3, as demonstrated previously (Zhang *et al*, 1999; Downes *et al*, 2000). In contrast to a sequential association of Sin3 and NuRD directed *in vivo* by OHT-bound ER α (Liu and Bagchi, 2004), Sin3 and NuRD complexes are combinatorially recruited by apo-ER α 46. This implies that OHT-bound ER α directs additional events to those engaged by apo-ER α 46, such as the recruitment of additional cofactors.

New insights into a 'transcriptional clock'?

We recently proposed that a 'transcriptional clock' synchronizes sequential waves of promoter accessibility for given cofactors to dictate an ordered sequence of events that initiate, achieve and then limit the transcriptional process. Cycles of apo-ER α isoforms binding to DNA give further insight into these mechanisms. First, comparison of the cycles directed by apo-ER α isoforms and those observed in the presence of E₂ (Métivier *et al*, 2003) indicates that

chromatin remodeling events affecting NucT are conserved. An initial stabilization into an 'active' conformation, followed by disruption of DNA-histone contacts around the TATA box occurs. The asymmetric flexibility of NucT that specifically occurs in MDA::ER α 66 cells correlates with acetylation of H3 K₁₄ and dimethylation of H4 R₃. Dimethylation of H4 R₃ destabilizes NuRD association with chromatin (Zegerman *et al*, 2002). NucT H4 R₃ dimethylation in MDA::ER α 66 may therefore limit the extent of NuRD action, which would otherwise result in a symmetric flexibility in NucT position as observed in MDA::ER α 46. Correspondingly, dimethylation of H4 R₃ on NucE, phased in association with the ERE of the pS2 promoter, which specifically occurs in MDA::ER α 66 cells, may explain the asymmetric 3' flexibility seen only in these cells. In the cycles described in this report, H4 K₁₆ and H3 R₁₇ neither become, respectively, acetylated nor dimethylated. This notwithstanding, variations in NucE positioning observed in MDA::ER α 46 at 30 min are likely generated by other histone acetylation events, as NucE variations are dependent on HDAC recruitment. However, these specific variations in NucE position observed in MDA::ER α 46 do not affect the permissiveness of the pS2 promoter to ER α , as both apo-ER α isoforms cycle with a periodicity of 20 min. Other examples of mechanisms linking the progress into the ordered recruitment of transcriptional complexes and chromatin modifications arise from the flexibility of NucE and NucT positions. Indeed, the continued binding of TBP and the presence of modified histones in MDA::ER α 66 may stabilize nucleosomes in their favored positions. In contrast, the repeated presence of NuRD and HDACs in MDA::ER α 46 may make the stabilized positioning more dependent on the presence of ER α and TBP. The transition between apo- and E₂-liganded cycles (45 min frequency) relies on liganded ER α -mediated disruption of histone-DNA contacts near the ERE (Mtvier *et al*, 2003). The exact role of the successive asymmetric and symmetric positioning of NucE observed in MDA::ER α 46 has therefore to be determined.

Interplay between protein recruitment and modification may generate additional marks that define the position of the 'transcriptional clock'. As the apo-ER α cycles are transcriptionally unproductive, at least some phases of the 'clock' are not dependent on the activation of PolII. In particular, TBP has a central role in synchronizing the cycles of events mediated by both apo-ER α isoforms. Indeed, the pS2 promoter recruits TBP to become poised for transcription, and cycles of TBP binding on the pS2 promoter persist over three cycles of ER α association. This implies that the presence of TBP does not strictly depend on the engagement of an initiating transactivation, in contrast to processes determined in yeast (Katan-Khaykovich and Struhl, 2002). Finally, although a number of repressive mechanisms influence the binding of TBP to the TATA box (Gaston and Jayaraman, 2003), the cyclical repression provoked by apo-ER α 46 appears to be TBP independent as neither deacetylation of H3 K₁₄ present within NucT nor the asymmetric flexibility of NucE affect the presence of TBP on the pS2 promoter. Moreover, the initial recruitment of a few general transcription factors signifies that a specific event takes place when ER α is liganded to E₂, leading to the recruitment of the complete transcriptional machinery. An as yet unknown mechanism therefore controls the engagement of TF_{II}B and

PolII: if this event does not occur, the cycle terminates and apo-ER α is cleared from the promoter. As proposed by Malik *et al* (1998), the PC4 component of the USA complex may be involved in this restriction of transcriptionally unproductive cycles. Recruitment of the proteasome through APIS, as described previously for ER α 66-mediated cycling (Reid *et al*, 2003), would also offer such a mechanism. However, mechanisms inducing APIS action to limit transcriptional activation remain unclear. Furthermore, the proteasome-mediated degradation of apo-ER α 46 in the cycles we describe has to be confirmed. The importance of TBP in the pS2 promoter cycles also raises questions with regard to the behavior of TATA⁻/CCAAT⁺ promoters that exhibit specific properties in terms of activator persistency and HAT recruitments (Carette *et al*, 2003).

α -Amanitin versus normal conditions, physiological importance of these cycles

Although key in the elucidation of the cyclical binding of apo-ER α isoforms onto DNA, the use of α -amanitin may bias the application of some of our findings to physiological conditions. The first issue relies on the exact role ER α has in the generation of the basal activity of the pS2 gene, which is abrogated by α -amanitin and reappears 2 h after α -amanitin release. Although necessary (Figure 1), the expression of ER α is unlikely to be directly involved in the processes leading to the activation of PolII in the absence of ligand. Indeed, apo-ER α does not have the structural capacity to recruit directly transcriptional activators such as proteins of the TRAP/DRIP family (Kang *et al*, 2002). Rather, our data indicate that apo-ER α induces, through binding to its responsive element and by limited cofactor interactions, a chromatin environment that is permissive for transcription to occur. This remodeling allows other transcription factors to induce transcription of the pS2 gene. In accordance with experiments performed by others (Barkhem *et al*, 2002), we determined these factors to include at least AP1 (Supplementary Figure S1). Furthermore, binding of apo-ER α and AP1 occurs on a different set of promoters (Supplementary Figure S1). Concordantly, the PolII signal appearing 2 h after α -amanitin treatment is kinetically independent of ER α . It follows that, in normal conditions, AP1 might act after that apo-ER α recruited some basal cofactors and induced a chromatin organization of the pS2 promoter adequate for AP1 actions. The functional synergism between ER α and AP1 observed when ER α is bound to estrogen (Barkhem *et al*, 2002) or when AP1 activity is stimulated (Bauer *et al*, 2002) would then be a separate mechanism. To gain further mechanistic details into this synergism, Re-ChIP experiments following the respective bindings of stimulated AP1 and E₂-bound ER α need to be performed. On the other hand, the engagement of apo-ER α proteins onto promoters provides the cell with mechanisms that enable a rapid adaptation of transcription to variations in the level of E₂. As transcription can be initiated by apo-ER α through phosphorylation events originating from growth factors signaling (Shao and Lazar, 1999), the interaction between apo-ER α and chromatin facilitates crosstalk between ER α and phosphorylation pathways. The nature and kinetics of events underlying this switch between transcriptionally unproductive and productive apo-ER α activities now have to be determined.

The second question relies on the ER α 46-specific repressive events, the mechanistic description of which required an initial closure of the promoter induced by the α -amanitin treatment. Therefore, ER α 46-specific repressive processes are maintaining rather than generating the repressed state of the pS2 promoter. To rule out definitely a potential active role for ER α 46 in generating a repressed pS2 promoter, experiments using inducible expression of ER α 46 will have to be carried out. The observation that apo-ER α 46 directs cyclical poisoning/inhibition of the pS2 promoter is one of the main outcomes of this work and introduces dynamic notions to the mechanisms underlying the maintenance of a promoter in a repressed state: (i) they require ER α 46 to poise initially the pS2 promoter for transcription through recruitment of factors similar to those engaged through ER α 66; and (ii) these mechanisms are subsequently cyclical. Generalization of this observation to other full-length NRs and other transcription factors remains to be determined. Divergent influences of the apo-ER α isoforms on transcription of target genes are confirmed by whole-genome analysis of gene expression in breast cells (Penot *et al*, manuscript in preparation), and were observed in bone cells (Denger *et al*, 2001; McDougall *et al*, 2003). Critical deficiencies in ER α ^{−/−} mice expressing *de facto* an ER α 46-like isoform (Kos *et al*, 2002) have made it apparent that AF-1 is the major transactivation function of ER α (Pendaries *et al*, 2002), and especially in differentiated cells (Mrot *et al*, 2004). Specific roles of ER α 46 may be restricted to effects on the uterine response and endothelial production of NO through actions at the membrane level (Li *et al*, 2003). However, cell-specific regulation of ER α 66/ER α 46 ratio would also result in differential outcomes in terms of ER α -mediated transcription in both the absence and presence of hormone. Examples in normal and neoplastic tissues are yet restricted to the observation that the expression of the ER α 46 in breast tumors and uterus is associated with estrogen refractoriness (Faye *et al*, 1986; Jozan *et al*, 1991). Verification of this issue is therefore a critical step to consider now.

Materials and methods

Reagents and antibodies

α -Amanitin, MNase, hydroxyquinone, Na bisulfite and estradiol were purchased from Sigma (Taufkirchen, Germany), *Taq* polymerase was from Roche (Mannheim, Germany) and *Hpa*II from New England Biolabs (Frankfurt, Germany). Antibodies were either gifts or purchased from Advanced Immunochemicals (Freiburg, Germany), Affiniti Research (Exeter, UK), Santa Cruz (Heidelberg, Germany) or Upstate Biotechnology (Buckingham, England) (Mtvier *et al*, 2003). Oligonucleotides were synthesized by MWG GmbH (Ebersberg, Germany).

Cell culture and immunobiochemistry

Cells were maintained in DMEM (Sigma) supplemented with 5% fetal calf serum (FCS, Sigma) and antibiotics (Roche) at 37C under 5% CO₂. MDA::MB231 cells stably expressing either ER α isoforms were created using pCDNA3.1Hygro (Invitrogen, Karlsruhe, Germany) constructs. Clones were grown in DMEM containing 5% charcoal-stripped serum, antibiotics and hygromycin (0.8 mg/

ml). For imaging, cells were seeded on glass-bottomed dishes (MatTek Corp., Ashland, MA, USA), and nuclei stained with Hoechst33342 (Molecular Probes, Eugene, OR, USA). ER α isoforms were localized after cell fixation with methanol and acetone by an HC20 staining revealed with a Cy³-labeled antibody (Molecular Probes).

Methylation analysis

Genomic DNA was prepared as described by Nelson and Krawetz (1992). The methylation status of CCGG sites was analyzed through digestion of 5 g of DNA with 10 U of *Hpa*II for 2 h at 37C. Once purified, digested DNA was subject to PCR using the following primer sets: A5', TGATTCTCCTGACTTACCCTCC; A3', CACGCTGTAATCCCAACACTTTT; B5', ATGGGCTTCATGAGCTCC; B3', AGGGTAAATACTGTACTAC; C5', CAAGATGACCTCACCACATG; C3', CGCAGATCACCTTGTCTCCAT; D5', TCCTTAGGCAAATGTTATCTAACG; and D3', GAGCGTTAGATAACATTTGCC. Bisulfite modification was performed on 5 g of DNA denatured in 0.3 M NaOH in 20 l for 30 min at 37C. A measure of 280 l of a 6.24 M urea/4 M bisulfite solution and 2 l of 100 mM hydroxyquinone was then added before an overnight incubation at 50C. Modified DNA was purified using the Qiaex II gel extraction kit (Qiagen, Hilden, Germany), denatured in 0.3 M NaOH for 5 min and precipitated. DNA was then subjected to nested PCR using the following primers: 5'a, TTAGAGTAGTTAGGATTATAGG; 5'b, GTTATGTTGGTTAGGT; and 3', TTACCTCCTCTCTACTCCAAA. PCR products were purified using the Qiaquick PCR purification kit (Qiagen), and sequenced.

PCRs

Sequences of the oligonucleotides used are: −701, TGATTCTCCTGACTTACCCTCC; −517, CACGCTGTAATCCCAACACTTTG; −350, GTTGTGACAGGCCAAGCCTTTT; −30, GAGCGTTAGATAACATTTGCC; −430, ATTAGCTTAGGCCTAGAC; −409, ATGGGCTTCATGAGCTCC; −362, CCAGTGTGTGACAGGCCAAGC; −285, CTGCAGAAGTGATTCTA; −266, AGGGTAAATACTGTACTAC; −245, TACTCATATCTGAGAGCCCT; −266, CTGGCGGGAGGGCCTCTCAGA; −167, CTGAGGATCTGAGATTCA; −190, GAAAGAGGGACTTTCTGAATC; −167, CAAGATGACCTCACCACATG; −129, TCTGTCTATCAGCAAAT; −52, GCGTTAGATAACATT; −23, GGATTTTATAGGGCAG; +20, TTGCC TCCTCTCTGCTCCAAA; pS2-5', ATGGCCACCATTGGAGAACA; pS2-3', TAAAACAGTGGCTCTCGGCG; GAPDH-5', TCTGTGTAAGTGATATTGTTG; and GAPDH-3', GATGGTGATGGGATTTC. Quantitative PCRs were performed using a SmartCycler (Eurogentec, Seraing, Belgium) or an ABI Prism^{} 7000 apparatus (Applied Biosystems).

ChIPs and Re-ChIPs

ChIP assays using 2 × 10⁶ cells synchronized by 3 days of culture in DMEM/2% dextran-charcoal-treated FCS cells and then treated with 2.5 M α -amanitin for 2 h followed by exposure to 10^{−8} M E₂ or ethanol were performed as described (Mtvier *et al*, 2003).

Preparation of mononucleosomes

Mononucleosomes were prepared from 5 × 10⁵ cells by MNase digestion following the method presented previously (Mtvier *et al*, 2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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