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Assembly in Breast Cancer

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## INTRODUCTION

Estrogen plays a critical role in the development and progression of breast cancer. While endocrine therapies play an important part in breast cancer treatment, the failure of these therapies reflects a lack of knowledge concerning the molecular mechanisms involved in estrogen signaling. The biological activities of estrogen are mediated by estrogen receptors (ER). In addition, a large number of proteins termed cofactors are involved in ER signaling. Until recently, our knowledge regarding these cofactors was based on their ability to bind receptors *in vitro* and affect transcriptional activation in transfection experiments. The *in vivo* role of these cofactors and the specific target genes involved in breast cancer are not well known. Therapeutic agents, such as tamoxifen, also bind ER, but block proliferation in breast cells. However, tamoxifen increases the risk of endometrial cancer. We have used chromatin immunoprecipitation (ChIP) to investigate cofactor involvement in ER signaling *in vivo* and to understand the mechanisms underlying the different actions of tamoxifen in breast and endometrial cells. We are in the process of using ChIP to identify the set of genes regulated by ER and its cofactors in these tissues. The detailed understanding of tissue- and ligand-dependent changes in gene expression gained through these studies will lead to more effective therapies for ER-dependent breast cancer.

## BODY

(Note: In June 2002 this award was transferred from the original Principal Investigator, Dr. Yongfeng Shang, to Dr. Eli Hestermann. This report represents the work of Dr. Shang in accomplishing Tasks 1 and 2 in the period July 2001 – April 2002 and Dr. Hestermann in beginning Task 3 in June 2002. Dr. Hestermann has prepared the report.)

Task #1 To identify the coactivators that are involved in the estrogen-induced transcription complex and to determine the sequence of events and the dynamics involved in the assembly and disassembly of the transcription complex

- a) Optimize chromatin immunoprecipitation (ChIP) assay, including PCR primer design and PCR reaction
- b) Test all antibodies for their feasibility for immunoprecipitation
- c) Perform ChIP at different times of estrogen treatment using antibodies against ER and coactivators

Status: Complete (see Shang *et al* 2000 *Cell* 103:843-852; Appendix A)

Task #2 To compare the protein components of the tamoxifen-induced ER complex that occupies estrogen-responsive gene promoters in breast cancer cells and in endometrial cancer cells

- a) Identify coactivators/corepressors that participate in tamoxifen-induced ER complex formation at target gene promoters in breast cancer cells
- b) Identify coactivators/corepressors that participate in tamoxifen-induced ER complex formation at target gene promoters in endometrial cancer cells
- c) Define the sequence of events that are involved in the assembly and disassembly of tamoxifen-induced ER complexes in both breast and endometrial cancer cells

Status: Objectives (a) and (b) accomplished for a subset of genes (see Shang and Brown 2002 *Science* 295:2465-2468; Appendix B)

These objectives are being pursued for additional estrogen targets in order to identify genes involved in cancer promotion. The mechanisms of complex assembly and disassembly (objective c) are being pursued.

Task #3 To identify new estrogen- and tamoxifen-responsive genes in breast cancer cells and endometrial cancer cells

- a) Isolate DNA fragments by ChIP and identify ER-regulated genes using microarrays.
- b) Confirm the targets by quantitative RT-PCR of RNA from estrogen- and

tamoxifen-treated breast and endometrial cells

- c) Apply ChIP using antibodies for coactivators and corepressors to identify the components of ER complexes at each gene
- d) Identify common and unique targets of ER in breast and endometrium

Status: In progress.

Task 3 was modified from the original proposal based on preliminary results. This modification was reflected in the revised Statement of Work submitted with the P.I. change. Originally, we had proposed to clone ChIP fragments directly and sequence them to identify responsive genes. However, we have subsequently developed a quantitative ChIP assay utilizing real-time PCR of known estrogen-responsive promoters. This assay shows that the enrichment of specific promoters in the ChIP sample versus input is about 10 to 20-fold (Figure 1). . Since ER-regulated genes constitute a miniscule proportion of total genomic DNA, this relatively modest enrichment means that a large majority of the DNA recovered by ChIP will be non-specific fragments. Therefore, cloning, sequencing, and screening these fragments would not be the most efficient use of materials and labor.

For this reason, I propose an alternative approach for identifying novel targets of ER action. The DNA from ChIP will be amplified and labeled by ligation-mediated PCR with fluorophore-conjugated nucleotides. The labeled DNA will then be hybridized to microarrays containing promoters of thousands of genes. Regulation of novel genes by estrogen and/or tamoxifen will be confirmed by measuring changes in gene expression by quantitative real time PCR. Such an approach has been used to identify genes regulated by E2F transcription factors in human cells (Ren *et al* 2002 *Genes Dev.* 16:245-56). Dr. Richard Young at the Whitehead Institute for Biomedical Research has agreed to collaborate on this project and provide the microarrays.

This will allow me to take the approach developed by Dr. Shang and expand it from the handful of genes that he has characterized to dozens or even hundreds of genes in both breast and endometrium. Work completed in Task 2 has revealed variations in cofactor recruitment and complex assembly on different genes in breast and endometrium. After novel targets have been identified in Task 3, I will determine the coactivators and corepressors involved in ER-dependent regulation of these genes as well.

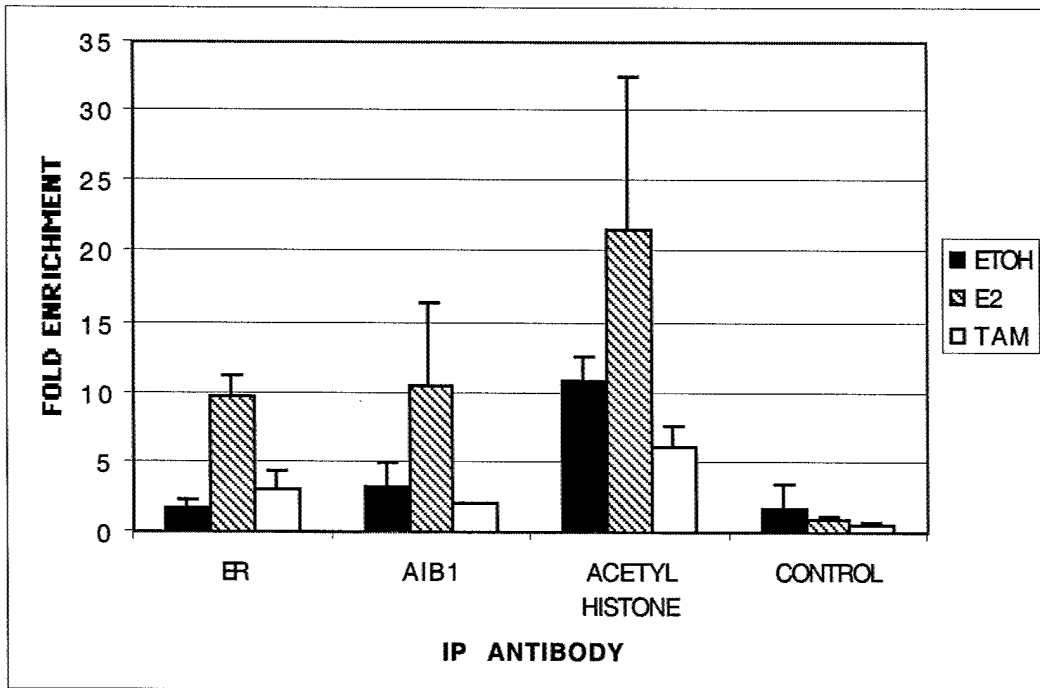


Figure 1. Enrichment of ER-responsive pS2 promoter by ChIP. MCF-7 cells were treated with ethanol (ETOH), estradiol (E2) or 4-hydroxytamoxifen (TAM) for 45 minutes, and then chromatin immunoprecipitation was performed as described previously (Shang et al 2000). Quantitative real time PCR was performed on the resulting DNA using primers specific for the estrogen response element in the pS2 promoter. Amounts of promoter were normalized to DNA input and represent enrichment over input (genomic) DNA.

## KEY RESEARCH ACCOMPLISHMENTS

- The ChIP assay has been optimized for estrogen receptor and its cofactors at known ER-responsive promoters.
- Several appropriate antibodies have been identified for IP
- The time-course of association of ER and cofactors at promoters has been measured and correlated to gene regulation, demonstrating that these factors associate and dissociate from promoters in a cyclic fashion
- Components of the ER complex have been identified in breast and endometrial cells
- Differences in cofactor expression between breast and endometrium have been linked to tissue-specific gene expression patterns
- A quantitative ChIP assay has been developed

## REPORTABLE OUTCOMES

Two publications: 1. Shang *et al* 2000 *Cell* 103:843-852  
2. Shang and Brown 2002 *Science* 295:2465-2468

Drs. Shang, Hestermann, and Brown have presented this work at several conferences, including the American Association for Cancer Research and the Nuclear Receptor Keystone meeting.

Based in large part on the success of this work, Dr. Shang was offered faculty positions at institutions both in the United States and abroad, and accepted a position at Beijing University.

## CONCLUSIONS

Progress to date represents a strong start in achieving the goals set forth in the statement of work. Identification and quantification of the binding of estrogen receptor and associated cofactors to promoters *in vivo* has provided novel insights into the mechanisms of gene regulation by ER. A key finding was that differential expression of the cofactor SRC-1 between breast and endometrium accounts for the action of tamoxifen in blocking proliferation in the former cells, while promoting proliferation in the latter. This finding should provide means for combating resistance to tamoxifen therapy in breast cancer treatment, as well as leading to mechanistic testing of novel therapeutic agents which are superior to tamoxifen for breast cancer prevention.

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Shang, Y. and M. A. Brown. (2002) Molecular determinants for the tissue specificity of SERMs. *Science* 295:2465-2468.

# Cofactor Dynamics and Sufficiency in Estrogen Receptor–Regulated Transcription

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## Summary

Many cofactors bind the hormone-activated estrogen receptor (ER), yet the specific regulators of endogenous ER-mediated gene transcription are unknown. Using chromatin immunoprecipitation (ChIP), we find that ER and a number of coactivators rapidly associate with estrogen responsive promoters following estrogen treatment in a cyclic fashion that is not predicted by current models of hormone activation. Cycles of ER complex assembly are followed by transcription. In contrast, the anti-estrogen tamoxifen (TAM) recruits corepressors but not coactivators. Using a genetic approach, we show that recruitment of the p160 class of coactivators is sufficient for gene activation and for the growth stimulatory actions of estrogen in breast cancer supporting a model in which ER cofactors play unique roles in estrogen signaling.

## Introduction

Estrogen plays an important role both in reproductive physiology and in numerous human disease states, including breast and endometrial cancers, cardiovascular disease, osteoporosis, and Alzheimer's disease. The biological actions of estrogen are mediated by the products of two genes within the nuclear receptor family, estrogen receptor (ER)  $\alpha$  and  $\beta$ . Current models of ER action suggest that it modulates the rate of transcription initiation through interactions with the basal transcription machinery and through alterations in the state of chromatin organization at the promoter of target genes via the recruitment of a variety of coactivators.

The assortment of coactivator proteins implicated in estrogen signaling includes three distinct but related p160 family members, SRC-1, TIF2 or GRIP-1, and AIB1 (also referred to as ACTR, RAC3, pCIP, or NCoA-3) (Onate et al., 1995; Anzick et al., 1997; Chen et al., 1997; Hong et al., 1997; Li et al., 1997; Torchia et al., 1997) and the histone acetylases CBP, p300 (Chakravarti et al., 1996; Hanstein et al., 1996) and the p300/CBP-asso-

ciated factor, pCAF (Blanco et al., 1998). Coactivators such as CBP, p300, pCAF, and possibly SRC1 and AIB1 possess intrinsic histone acetyltransferase (HAT) activities capable of modifying the chromatin organization of the target gene promoters. However, the participation of all of these proteins with a common enzymatic activity in ER transactivation raises the question of functional redundancy. In addition a distinct multiprotein complex first found to be involved in thyroid hormone receptor (TR) and vitamin D receptor (VDR) signaling (Fondell et al., 1996; Rachez et al., 2000) has also been implicated recently in ER action through an interaction with its PBP/TRAP220/DRIP205 subunit (hereafter PBP) (Burakov et al., 2000).

Much of our current knowledge regarding the involvement of these proteins in nuclear receptor signaling is based on their ability to bind liganded-receptors in vitro and enhance transcriptional activation in transfection experiments. Although the participation of so many protein factors in ER-mediated gene transactivation undoubtedly reflects the complexity of the transcription in eukaryotes, it also raises the question of which if any of these coactivators are necessary and/or sufficient for the transcriptional activation of ER in vivo. In addition, the transcriptional dynamics of ER action are also not well understood. The traditional view that activators such as ER bind to a response element in the promoter of a target gene and remain associated for as long as the stimulus is present (Hahn, 1998; Berk, 1999) is inconsistent with a recent report that the association of ER and AIB1 is a transient process that is disrupted by acetylation of AIB1 by CBP/p300 (Chen et al., 1999). This raises the question of whether the ER complex might cycle on and off the target gene promoters.

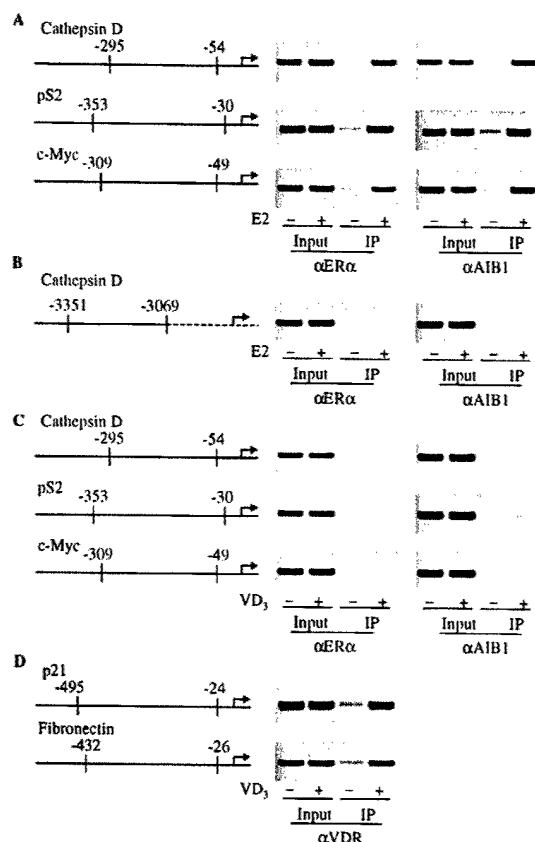
We have addressed these critical questions under biologically relevant conditions through the study of endogenous ER $\alpha$  and cofactors in breast cancer cells. Using chromatin immunoprecipitation (ChIP) we find that in response to estrogen native ER transcription complexes are stimulated to assemble on target promoters in a cyclic fashion. These experiments demonstrate differential roles for the various coregulators in the assembly of ER transcription complexes in vivo. In addition we demonstrate that TAM-bound ER recruits corepressors but not coactivators to target promoters suggesting that the cellular responses to a selective ER modulator (SERM) such as TAM may reflect the balance between coactivators and corepressors present in different cells. Finally, using a p160 coactivator that binds ER with reversed ligand specificity, we show that recruitment of this class of coactivator is sufficient for gene activation as well as estrogen-dependent cell cycle progression.

## Results

### Estrogen Induces Occupancy of Estrogen Target Gene Promoters by ER $\alpha$ and p160 Coactivators

To investigate the assembly of the ER transcription complex, we first examined the recruitment of ER $\alpha$  and p160

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**Figure 1. The Recruitment of ER $\alpha$  and AIB1 to the Promoters of Estrogen-Responsive Genes**

(A) Soluble chromatin was prepared from MCF-7 cells treated with E2 for 45 min and immunoprecipitated (IP) with antibodies against ER $\alpha$  ( $\alpha$ ER $\alpha$ ) or against AIB1 ( $\alpha$ AIB1). The final DNA extractions were amplified using pairs of primers that cover the regions of cathepsin D, pS2 and c-Myc gene promoters as indicated. (B) The distal region of the cathepsin D gene promoter was examined for the presence of ER $\alpha$  and AIB1. (C) MCF-7 cells were treated with 1,25-(OH) $_2$ D $_3$  (VD $_3$ ) for 45 min and the cathepsin D, pS2, and c-Myc gene promoters were examined for the occupancy by ER $\alpha$  or AIB1 using the same antibodies and primers as in (A). (D) MCF-7 cells were treated with 1,25-(OH) $_2$ D $_3$  (VD $_3$ ) for 45 min and the occupancy of the vitamin D receptor (VDR) on the p21 and fibronectin gene promoters was examined.

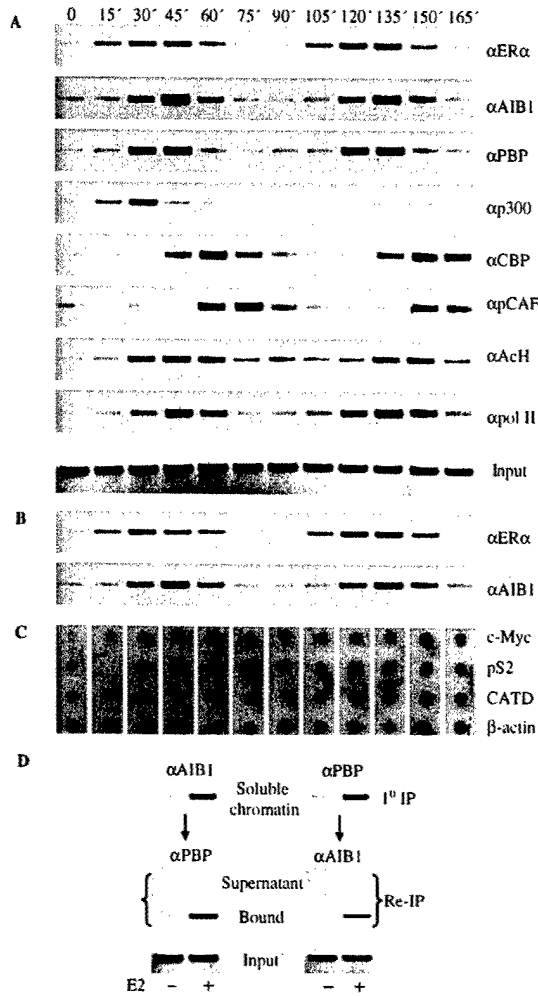
coactivators to the promoters of endogenous estrogen-responsive target genes following estrogen treatment. The estrogen-dependent human breast cancer cell line MCF-7 was used for these studies as there are several well-characterized estrogen target genes in these cells. Three estrogen target genes, cathepsin D (CATD), pS2, and c-Myc (Dubik and Shiu, 1992; Augereau et al., 1994; Giamarchi et al., 1999) were examined. MCF-7 cells were grown in the absence of estrogen for at least three days followed by either no treatment or treatment with saturating levels of 17 $\beta$ -estradiol (E2) for 45 min. The status of the endogenous transcription complexes present on the estrogen responsive regions of these promoters was determined using chromatin immunoprecipitation (ChIP). The presence of the specific promoters in the chromatin

immunoprecipitates was analyzed by semiquantitative PCR using specific pairs of primers spanning the estrogen responsive regions in the three promoters. As shown in Figures 1A, treatment with E2 induced a dramatic increase in the occupancy by both ER $\alpha$  and AIB1 of the CATD, pS2 and c-Myc gene promoters. Similar promoter occupancy by the other p160 family members SRC-1 and GRIP1 was also observed (data not shown). As expected, given the ~500 bp to ~2000 bp size of the DNA fragments produced by sonication in these experiments, PCR analysis did not detect any significant increase in ER $\alpha$  or AIB1 occupancy of a region ~3 kb upstream of the CATD promoter (Figure 1B). In addition, treatment of MCF-7 cells under the same conditions with 1,25-(OH) $_2$ D $_3$  (VD $_3$ ) failed to induce any increase in ER $\alpha$  or AIB1 association with these promoters (Figure 1C), although VD $_3$  treatment did result in an increased occupancy of the VD $_3$  receptor (VDR) on the promoters of VD $_3$  responsive p21 (Liu et al., 1996; Verlinden et al., 1998) and fibronectin (Polly et al., 1996) genes (Figure 1D).

#### Dynamics of ER Transcription Complex Assembly

Having shown that estrogen is able to induce occupancy of responsive promoters by ER $\alpha$  and AIB1, we sought to understand the precise order and timing of complex assembly using ChIP. Strikingly, ER $\alpha$  is recruited to the CATD promoter within 15 min following the addition of E2 (Figure 2A). ER $\alpha$  promoter occupancy peaks at 30–45 min and returns to baseline by 75 min. To further validate these findings, we designed a highly quantitative ChIP assay using real-time PCR and an ABI PRISM 7700 Sequence Detector (Perkin-Elmer). Quantitative ChIP was performed to determine the relative levels of CATD promoter occupancy by ER $\alpha$  following treatment of MCF-7 cells with E2. The measured level of CATD promoter occupied by ER $\alpha$  increased ~50-fold within 15 min following the addition of E2. Maximal induction of promoter occupancy of greater than 100-fold was detected at 30 min and this returned almost to baseline at later times. Thus the differences observed by ChIP in Figure 2A reflect very significant quantitative changes in promoter occupancy and confirm that ER $\alpha$  cycles onto and off of the CATD promoter in response to E2. In order to rule out epitope masking as an alternate explanation for the apparent cycling of ER $\alpha$ , we used multiple monoclonal antibodies directed toward different ER $\alpha$  epitopes in the ChIP assay. Three different monoclonal antibodies gave almost identical patterns of ER $\alpha$  association with the CATD promoter suggesting that epitope masking is an unlikely explanation for the observed cycling (data not shown).

We next sought to determine the participation and timing of association of the various coactivator proteins in the formation of the ER $\alpha$  transcription complex, and the relationship of this to the state of histone acetylation and the onset of gene transcription. As was observed for ER $\alpha$ , the coactivators AIB1, PBP, and p300 all rapidly and transiently associate with the CATD promoter as significant CATD promoter occupancy is observed within 15–30 min following the addition of E2. Concurrent with this first wave of factor association, histones associated with the CATD promoter are acetylated. This is closely followed by the association of RNA pol II. CBP



**Figure 2.** The Dynamics of ER $\alpha$  Transcription Complex Assembly (A) Occupancy of the cathepsin D (CATD) promoter by ER $\alpha$ , different coactivators, acetylated histones (AcH) and RNA polymerase II (pol II) at different times as measured by ChIP. (B) Occupancy of the pS2 promoter by ER $\alpha$  and AIB1 as measured by ChIP. (C) Nuclear run-on analysis of the expression of c-Myc, pS2, CATD, and  $\beta$ -actin mRNA in MCF-7 cells treated with E2 for various times. (D) ChIP Re-IP to examine whether the ER $\alpha$ -PBP complex and ER $\alpha$ -AIB1 complex are assembled on the same promoters. Soluble chromatin was prepared from MCF-7 cells treated with E2 for 40 min and divided into two aliquots. One aliquot was first immunoprecipitated with antibodies against AIB1 (1<sup>o</sup> IP). The supernatant was collected and reimmunoprecipitated with antibodies against PBP (Supernatant Re-IP). The other aliquot was first immunoprecipitated with antibodies against PBP (1<sup>o</sup> IP) followed by reimmunoprecipitation with antibodies against AIB1. Similar reciprocal Re-IPs were also performed on complexes eluted from the 1<sup>o</sup> IPs (Bound Re-IP).

and pCAF also become transiently associated with the same region of the CATD promoter but only beginning at about 45 min following the onset of E2 stimulation. This is a time when association of the first wave of factors is already falling suggesting that CBP and pCAF function at a distinct step in the process of ER $\alpha$ -mediated activation.

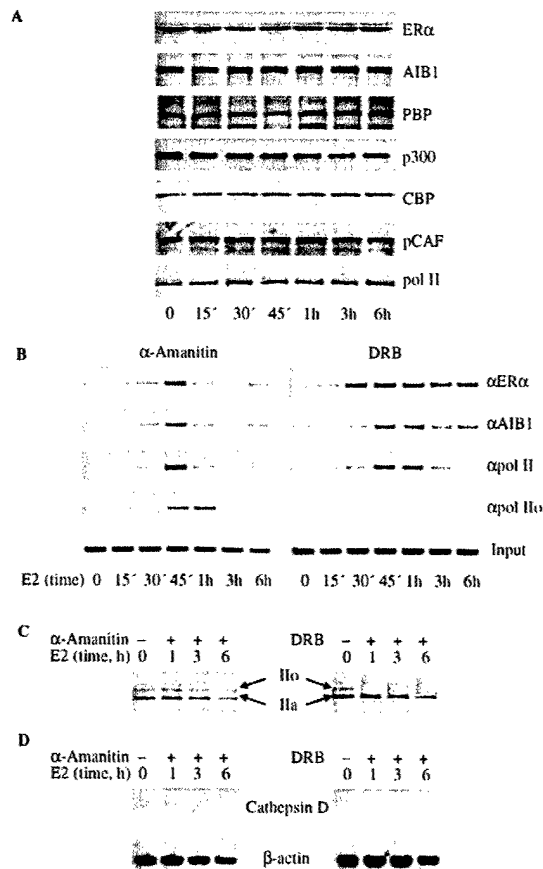
Notably, the second cycle of promoter occupancy

beginning about 100 min following estrogen stimulation differs somewhat from the first. While ER $\alpha$ , AIB1, PBP, CBP, pCAF all assemble in the same order and with the same timing as in the first cycle, p300 is not detected in the second cycle of complex assembly. Interestingly, while a second increase in the level of acetylated histones associated with the CATD promoter is detected during the second cycle, this is from a baseline level that remains elevated between the first and second cycle of complex assembly.

In order to confirm that the repeated cycling of the ER $\alpha$  complex was not unique to the CATD promoter in MCF-7 cells, we examined in detail the association of ER $\alpha$  and AIB1 with the pS2 promoter (Figure 2B). As was seen on the CATD promoter, both ER $\alpha$  and AIB1 repeatedly cycle onto and off of the pS2 promoter with very similar dynamics. This suggests that the cyclic nature of ER $\alpha$  complex assembly may be a general property of ER $\alpha$ -regulated genes. In addition, to determine whether the cyclic nature of the recruitment of the ER transcription complex to the CATD promoter is restricted to MCF-7 breast cancer cells, similar time courses were performed in ECC-1 endometrial cancer cells. Like MCF-7, ECC-1 cells express ER $\alpha$  and are E2 responsive for CATD expression and growth (Castro-Rivera et al., 1999). A very similar pattern of ER $\alpha$  and AIB1 recruitment to the CATD promoter was seen in ECC-1 as was seen in MCF-7 (data not shown). Whether the details of the factors involved and the timing of their assembly differ in a promoter- and/or cell type-specific manner is an intriguing possibility that remains to be determined.

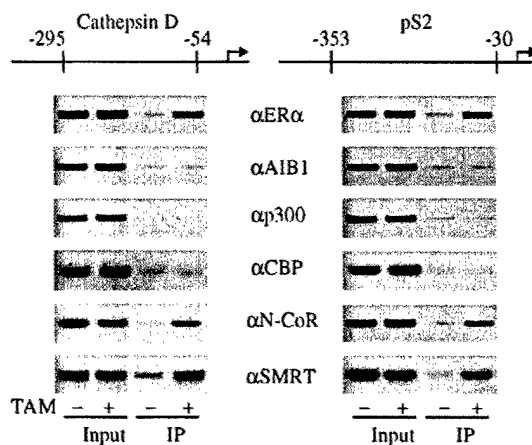
To assess when the ER $\alpha$  transcription complex becomes competent for gene activation, we examined whether the repeated cycles of complex assembly are followed by transcription. Nuclear run-on assays were performed on the c-Myc, pS2, and CATD genes following estrogen stimulation of MCF-7 cells (Figure 2C). Significant transcription is evident after 45 min of estrogen stimulation. This follows the assembly of ER $\alpha$ , PBP, AIB1, p300, and pol II on the promoter and precedes the association of CBP and pCAF. Interestingly, as is seen with assembly of ER $\alpha$  and its associated cofactors on the promoter, transcription is also cyclic. These results confirm that the cyclic assembly of the ER $\alpha$  complex on the promoter is followed by cycles of transcription.

Additionally, the time course of coactivator recruitment reveals important aspects of the function of two distinct coactivator complexes. PBP, the protein that anchors the DRIP/TRAP complex to ER $\alpha$  and to other nuclear receptors (Rachez et al., 1998, 1999, 2000; Burakov et al., 2000) is recruited to the CATD promoter at the same time as ER $\alpha$  and AIB1. This result rules out a sequential model and supports either a combinatorial model in which the PBP and AIB1 containing complexes act simultaneously on the very same CATD promoters or a parallel model in which the two complexes are being recruited to distinct subsets of CATD promoters on different alleles or in different cells. In order to test whether a combinatorial or a parallel model applies, we performed a serial ChIP experiment (Figure 2D). For this we divided the soluble chromatin derived from E2-treated or untreated cells into two aliquots. One was immunopre-



**Figure 3. Mechanism of ER $\alpha$  Transcription Complex Disassembly**  
(A) Protein levels of the components of the ER $\alpha$  transcription complex in MCF-7 cells at different times following estrogen treatment measured by Western blotting. (B) Recruitment patterns of ER $\alpha$ , AIB1, RNA polymerase II (pol II) and phosphorylated RNA polymerase II (pol Ilo) on the cathepsin D promoter in MCF-7 cells treated with  $\alpha$ -amanitin (left panel) or DRB (right panel) followed by E2 for different times. (C) Western blot analysis of the phosphorylation status of RNA polymerase II after treatment with  $\alpha$ -amanitin (left panel) or DRB (right panel) followed by E2 for various times. Both underphosphorylated (Ila) and hyperphosphorylated (Ilo) forms of the large subunit of RNA polymerase II are shown. (D) Inhibition of transcription by  $\alpha$ -amanitin (left panel) and DRB (right panel) was confirmed by Northern blotting in MCF-7 cells for the expression of the CATD mRNA.  $\beta$ -actin serves as a loading control.

cipitated with AIB1 antibodies followed by release of the immune complexes and reimmunoprecipitated (Re-IP) with PBP antibodies. The other was first immunoprecipitated with PBP antibodies followed by release and Re-IP with AIB1 antibodies. The same Re-IP was also performed on the unbound supernatant fractions from the primary immunoprecipitation. While both AIB1 antibodies and PBP antibodies were able to immunoprecipitate the CATD promoter after cells were treated with E2 (Figure 2D), subsequent supernatant Re-IPs with either PBP antibodies or AIB1 antibodies were unable to do so. On the other hand, subsequent Re-IPs of the eluted primary immunoprecipitates were able to bind the CATD promoter ("bound" in Figure 2D). These experiments



**Figure 4. Promoter Occupancy by ER $\alpha$  and Cofactors Induced by Tamoxifen (TAM)**  
(A) ChIP demonstrates the promoter occupancy by ER $\alpha$  and the indicated cofactors on the CATD (left panel) and pS2 (right panel) promoters from MCF-7 cells treated with TAM.

support a model in which AIB1 and PBP act in a combinatorial fashion on the same ER $\alpha$  responsive promoter.

#### Phosphorylation of pol II Is Required for ER Complex Cycling

Our data show that the association of ER $\alpha$  and the other components of the transcription initiation complex with the promoter is cyclical. This raises the question of what events regulate the release of the complex from the promoter. We first examined whether the overall cellular levels of the factors change over the time course of estrogen stimulation. The levels of ER $\alpha$  and the other factors do not fluctuate significantly over the first hour of E2 treatment during which time they have cycled onto and off of the CATD promoter and stable levels are present for as long as 6 hr (Figure 3A).

To address whether release from the promoter occurs at a specific step during transcription initiation, two inhibitors of transcription,  $\alpha$ -amanitin and 5,6-dichloro-1- $\beta$ -D-ribofuranosylbenzimidazole (DRB) were used. MCF-7 cells were treated with either 10  $\mu$ g/ml of  $\alpha$ -amanitin or 50  $\mu$ M of DRB for 1 hr before the addition of E2, and CATD promoter occupancy was determined by ChIP for ER $\alpha$ , AIB1, and both total RNA pol II and its hyperphosphorylated Ilo form (Figure 3B).

$\alpha$ -Amanitin is able to bind to the large subunit of RNA pol II (Kedinger et al., 1970; Lindell et al., 1970) and block the incorporation of new nucleotides into the nascent RNA chain (de Marco et al., 1989). Interestingly  $\alpha$ -amanitin had no obvious effect on the pattern of CATD promoter occupancy by ER $\alpha$ , AIB1, and RNA pol II (Figure 3B, left panel) or the generation of the phosphorylated Ilo form of pol II (Figures 3B and 3C, left panel) though CATD transcription was significantly inhibited by  $\alpha$ -amanitin (Figure 3D, left panel). Thus, the assembly and subsequent release of the ER $\alpha$  transcription complex from the CATD promoter does not depend on the incorporation of nucleotides into the nascent RNA transcript.

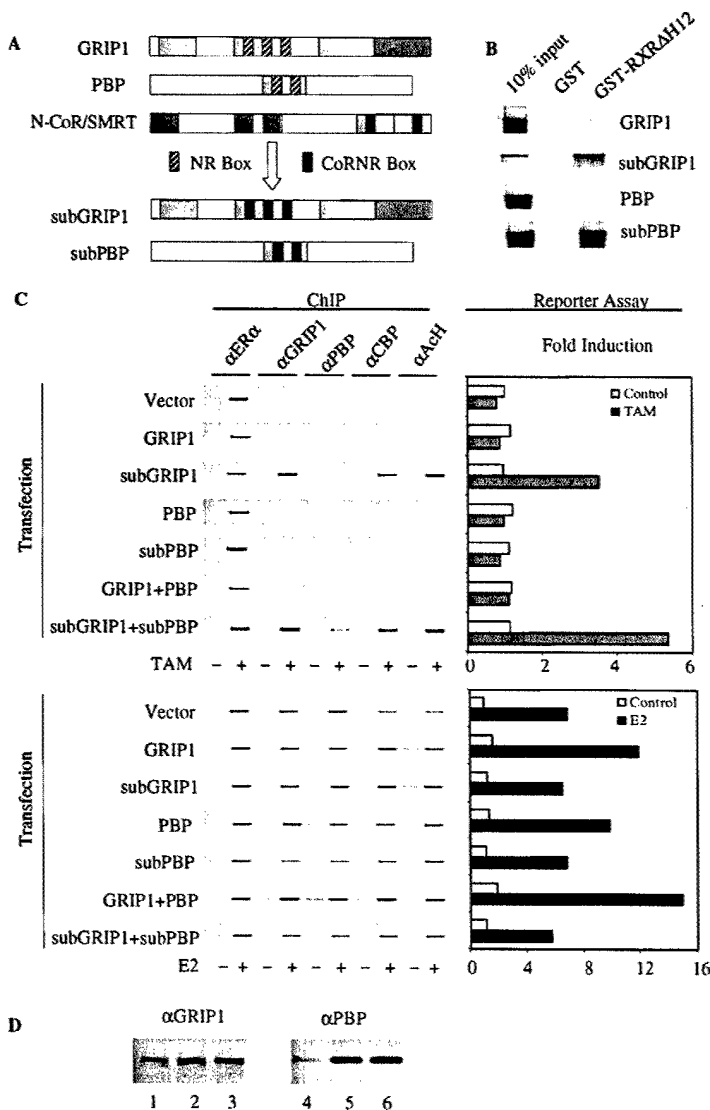


Figure 5. Central Role for p160 Proteins in the Assembly of the ER $\alpha$  Transcription Complex

(A) Diagram of the structure of subGRIP1 and subPBP. (B) Interaction of in vitro transcribed and translated GRIP1, PBP, subGRIP1, and subPBP with glutathione S-transferase (GST) or a GST fusion of helix 12 deleted RXR (GST-RXR $\Delta$ H12) was assayed by GST pull-down in the absence of ligand. (C) ChIP (left panel) and reporter (right panel) assays were performed on MCF-7 cells transfected with the indicated expression plasmids and treated with TAM (upper panel) or E2 (lower panel). The occupancy of the CATD promoter by ER $\alpha$ , GRIP1, CBP, PBP, and acetylated histone (AcH) was examined. (D) Western blotting analysis of protein expression in MCF-7 cells transfected with GRIP1 (lane 2) or subGRIP1 (lane 3), or transfected with PBP (lane 5) or subPBP (lane 6). Lane 1 and lane 4 are vector transfection controls.

DRB acts to block transcription by inhibiting CDK7 (Yankulov et al., 1995) and CDK9 (Marshall et al., 1996), two kinases responsible for phosphorylating the RNA pol II large subunit C-terminal domain. In contrast to what we found with  $\alpha$ -amanitin, DRB treatment led to the stabilization of ER $\alpha$ , AIB1, and RNA pol II on the CATD promoter for several hours (Figure 3B, right panel). As expected DRB also effectively blocked CATD transcription (Figure 3D, right panel) as well as the phosphorylation of pol II (Figures 3B and 3C, right panel). These results suggest that the release of the ER $\alpha$  transcription complex from the promoter requires the phosphorylation of the RNA pol II large subunit and are consistent with other data showing that the activity of RNA polymerase II is regulated by multisite phosphorylation on its C-terminal domain. Underphosphorylated RNA pol II C-terminal domain is believed to mediate multiple protein-protein interactions involved in the assembly of the preinitiation complex while the subsequent phosphory-

lation of the C-terminal domain contributes to the initiation of transcription and elongation of the primary transcript (Corden and Patturajan, 1997; Bentley, 1998). Taken together with the ability of the ER $\alpha$  transcription complex to be released from the promoter in the presence of  $\alpha$ -amanitin, we conclude that promoter release occurs subsequent to RNA pol II C-terminal domain phosphorylation and prior to or concurrent with transcription initiation.

#### Tamoxifen Induces the Formation of an ER-Corepressor Complex

Tamoxifen (TAM) competes with E2 for ER $\alpha$  binding and induces a conformational change in which the recruitment of p160 coactivators is blocked (Halachmi et al., 1994; Brzozowski et al., 1997). In addition, while it functions as an antagonist in breast cancer cells such as MCF-7, in other tissues and on certain promoters TAM acts as a partial ER $\alpha$  agonist. This property has led TAM

to be viewed as the prototypical selective ER modulator or SERM. To investigate further the mechanisms underlying the activity of TAM-ER $\alpha$ , we examined the recruitment of coactivators or corepressors in MCF-7 cells after treatment with TAM (Figure 4). As was observed with E2, TAM treatment induced ER $\alpha$  occupancy of the CATD (Figure 4, left panel) and pS2 promoters (Figure 4, right panel). As expected, the TAM-ER $\alpha$  complex did not recruit p160 coactivators such as AIB1 nor CBP nor p300. In marked contrast, when we examined promoter occupancy by corepressors, we detected the recruitment of the nuclear receptor corepressors N-CoR and SMRT. These data show that in addition to inducing a conformational change in ER $\alpha$  that blocks coactivator recruitment, TAM is able to induce the recruitment of ER $\alpha$  and an associated corepressor complex to the promoter, suggesting TAM-ER $\alpha$  may be actively involved in gene repression. This also suggests that the ratio of agonism to antagonism seen with SERMs such as TAM may be influenced by the levels or activity of the corepressor complex.

**p160 Proteins Play a Central Role in the Assembly and Activity of the ER Transcription Complex**

PBP and the p160 coactivators utilize similar NR boxes that specifically recognize the agonist-bound conformation of ER $\alpha$ . The NR box is characterized by an LXXLL sequence flanked with a short stretch of amino- and carboxyl-terminal amino acids and is both necessary and sufficient for ligand-dependent interactions of p160 proteins and PBP with AF2 domains of nuclear receptors (Heery et al., 1997; Ding et al., 1998). Analogously, the two interaction domains of the nuclear receptor corepressors have been found to contain a conserved sequence referred to as the CoRNR box (Hu and Lazar, 1999) or as an LXXI/HIXXXI/L helix that recognizes the unliganded and repression competent form of thyroid and retinoid receptors (Nagy et al., 1999; Perissi et al., 1999). It has been suggested that the nuclear receptor AF-2 helix has evolved to discriminate between the NR box LXXLL helix in coactivators and the CoRNR box helix in the N-CoR/SMRT corepressors, permitting the ligand-dependent switch of nuclear receptor activity. In the case of ER $\alpha$ , this switch may be operated by agonist versus antagonist binding. In order to define the roles of p160 proteins and PBP in the assembly and activities of the ER transcription complex, we utilized chimeric coactivator/corepressor proteins in which the three NR boxes in GRIP1 and the two NR boxes in PBP had been replaced by CoRNR boxes (Figure 5A). These CoRNR box substituted cofactors termed subGRIP and subPBP are recruited to the unliganded and helix 12 deleted form of RXR *in vitro* while the wild-type proteins are not, confirming their reversed specificity (Figure 5B).

The substituted cofactors were expressed in MCF-7 cells either separately or together, and promoter occupancy after treatment with E2 or TAM was assessed by ChIP. When wild-type GRIP1 was expressed in MCF-7 cells, as expected, only ER $\alpha$  was recruited to the CATD promoter in response to TAM (Figure 5C, upper left panel). As control, when these cells were treated with E2, the recruitment of ER $\alpha$ , GRIP1, CBP, and PBP was observed (Figure 5C, lower left panel). In marked con-

trast, after introduction of subGRIP1 into MCF-7 cells, TAM was able to induce the recruitment of not only ER $\alpha$ , but also GRIP1 and CBP (Figure 5C, upper left panel). Remarkably, subGRIP1 transfection led to histone acetylation in response to TAM, suggesting that subGRIP1 was sufficient to induce an activated chromatin template on the CATD promoter. Transcriptional activation was confirmed by cotransfection of subGRIP1 with an estrogen-responsive luciferase reporter (Figure 5C, right panel). Importantly PBP was not recruited by TAM-ER $\alpha$  even in the presence of subGRIP1, suggesting that PBP recruitment is not a necessary component of an active ER $\alpha$  transcription complex.

To further assess the role of PBP in ER $\alpha$ -mediated gene activation, MCF-7 cells were transfected with either wild-type PBP or CoRNR box-substituted PBP, subPBP, and treated with TAM. As expected transfection of wild-type PBP did not facilitate the recruitment of PBP, GRIP1, or CBP in response to TAM. Surprisingly however, transfection of subPBP gave the same results suggesting that substitution of the CoRNR box for the NR box in PBP was not sufficient to promote its recruitment by TAM-bound ER $\alpha$  nor to allow gene activation (Figure 5C, top panels), even though in control experiments PBP recruitment and gene activation were observed in both PBP and subPBP-transfected cells treated with E2 (Figure 5C, lower panels). Interestingly, when both subGRIP1 and subPBP were introduced into cells, PBP was recruited to the ER $\alpha$  complex by TAM and the addition of subPBP increased the level of activation compared to that seen with subGRIP1 alone (Figure 5C, top panels). These differences were not due to differences in protein expression as measured by Western blotting (Figure 5D). Taken together these results suggest that while substitution of the CoRNR box for the NR box is sufficient for recruitment of p160 coactivators and ER $\alpha$ -mediated gene activation, the recruitment of PBP to the ER $\alpha$  complex requires both an interaction with ER $\alpha$  and the presence of a p160 factor.

To determine whether the sufficiency of p160 action

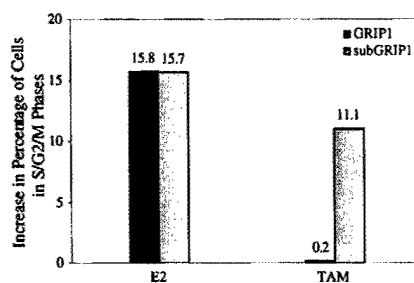


Figure 6. Induction of Cell Proliferation in MCF-7 Cells Expressing subGRIP1

MCF-7 cells were grown in DMEM supplemented with 10% charcoal-dextran-stripped fetal bovine serum for 24 hr and were cotransfected with a GFP expression plasmid together with either a GRIP1 or a subGRIP1 expression plasmid. Forty-eight hours after transfection, cells were treated with 100 nM E2 or 5  $\mu$ M TAM or untreated for another 16 hr. Cells were then collected and analyzed by flow cytometry. The numbers indicate the increase in the percentage of GFP-positive cells that are in the S/G2/M phases of the cell cycle after 16 hr treatment with E2 or TAM.

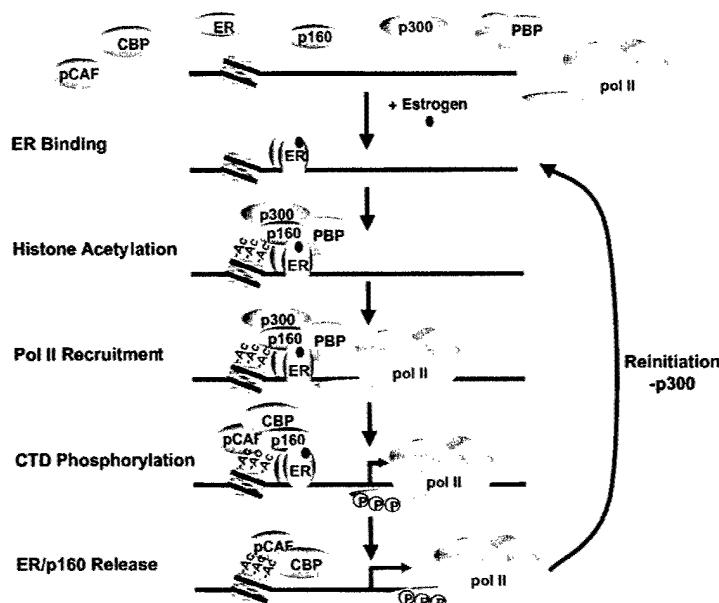


Figure 7. Cyclic Model of ER Transcription Complex Assembly

The sequential formation of complexes leading to the activation of gene expression by agonists such as estradiol. See text for details.

for gene activation extends to a physiologically relevant response in breast cancer cells, we examined the effects of TAM on cell cycle progression of MCF-7 cells expressing subGRIP1. Estrogen is normally required for G<sub>1</sub>/S transition of MCF-7 cells and estrogen deprivation leads to a significant G<sub>1</sub> arrest. We cotransfected estrogen-deprived MCF-7 cells with GRIP1 or subGRIP1 together with a green fluorescent protein (GFP) expression construct. Cells then were treated with E2 or TAM and the cell cycle profile of the GFP-expressing population was determined by flow cytometry (Figure 6). In E2-deprived MCF-7 cells expressing either wild-type GRIP1 or subGRIP1, ~85% of the GFP-expressing cells were arrested in the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. E2 addition for 16 hr was able to release ~15% of the GFP-expressing cells into cell cycle with the G<sub>0</sub>/G<sub>1</sub> fraction changing from ~85% to ~70%. Treatment of wild-type GRIP1-expressing cells with TAM for 16 hr had no effect on the cell cycle profile with ~85% of cells remaining in G<sub>0</sub>/G<sub>1</sub>. In marked contrast, in cells expressing subGRIP1, TAM treatment was able to effect the release of ~11% of the cells into the cell cycle. These results indicate that the recruitment by ER $\alpha$  of a p160 coactivator is sufficient to exert the cell proliferating properties of estrogen in breast cancer.

#### Discussion

##### Dynamics of ER Transcription Complex Assembly

Genetic and biochemical studies over the past two decades have revealed that the process of gene activation in eukaryotic cells is extremely complicated. Chromatin immunoprecipitation (ChIP) is a powerful technique that offers the advantage of being able to detect endogenous transcription factors bound *in vivo* to promoters under different physiologic conditions. In addition to direct promoter binding factors, the presence of proteins that are not bound directly to DNA and that depend on other

proteins for promoter binding can also be determined using ChIP. Using ChIP, we found that p160 coactivators, CBP, p300, pCAF, and PBP are recruited in a specific order to the ER transcription complex after estrogen stimulation in MCF-7 breast cancer cells.

Interestingly, the ER transcription complex appears to repeatedly cycle onto and off of target promoters in the presence of continuous stimulation by estrogen. The regular cycling of the ER $\alpha$  transcription complex may represent a mechanism that favors continuous sampling of the external milieu. Cycling may be regulated in part by covalent modification of coregulators (Chen et al., 1999; Font De Mora and Brown, 2000; Rowan et al., 2000). In addition our inhibitor studies suggest that the cycling of the ER $\alpha$  complex off the promoter depends on the phosphorylation of the C-terminal domain of the large subunit of RNA pol II. Factors that alter the phosphorylation of RNA pol II might affect the responsiveness of ER $\alpha$ -dependent promoters by interfering with the cycling of ER $\alpha$  complex.

We confirmed that TAM induces the recruitment of ER $\alpha$  to responsive promoters. We did not observe recruitment of coactivators in response to TAM in MCF-7 cells, where TAM is a full antagonist. Consistent with *in vitro* studies (Jackson et al., 1997; Smith et al., 1997; Lavinsky et al., 1998), TAM-bound ER $\alpha$  did recruit nuclear receptor corepressors N-CoR and SMRT to the promoters as well. Preliminary data suggests that TAM-ER $\alpha$  actively represses transcription of genes to which it recruits these corepressors (data not shown).

##### Functional Specificity and Sufficiency of Coactivators

Although several lines of evidence point to functional differences between CBP and p300, (reviewed by Glass and Rosenfeld, 2000), evidence for functional differences among CBP, p300, and pCAF in ER $\alpha$ -mediated transcription has been lacking. Our experiments show

that p300, CBP and pCAF are all involved in ER $\alpha$ -mediated gene transcription and that in the first cycle of transcription initiation these three proteins are sequentially recruited to an ER $\alpha$  transcription complex, with p300 first, followed by CBP and pCAF. These findings agree well with a previous observation that p300 interacts specifically with the nonphosphorylated, initiation-competent form of RNA polymerase II, whereas pCAF interacts with the elongation-competent, phosphorylated form (Cho et al., 1998). Our observation that p300 is not recruited in subsequent cycles is consistent with *in vitro* transcription data suggesting that while p300 plays a role in transcription initiation by ER $\alpha$ , it does not participate in reinitiation (Kraus and Kadonaga, 1998). These results may also indicate that histone acetylation and chromatin remodeling could be a step-wise process in which each of these three cofactors exerts a distinct and nonredundant role and each of these three HAT proteins exhibits a different substrate specificity, as suggested by *in vitro* studies (Schiltz et al., 1999).

The PBP/DRIP/TRAP complex has been proposed to represent a distinct complex from the ER $\alpha$ -p160 complex and to act at a later stage in gene activation, after histone acetylation by the p160 complex makes chromatin more accessible. Our data show that PBP is recruited to ER $\alpha$  responsive promoters rapidly after estrogen stimulation, at about the same time as p160 factors and p300. In addition, our ChIP Re-IP results argue that the p160 complex acts in combination with the PBP complex on the same ER $\alpha$  responsive promoter rather than the two complexes acting independently from each other on different promoters.

The reversed pharmacology of GRIP1 and PBP with CoRNR box substitutions allowed us to determine which of these coactivators are sufficient for binding and activation of an ER $\alpha$  complex. Our results indicate that recruitment of a p160 coactivator is sufficient to induce assembly of an ER $\alpha$  complex capable of gene activation without the recruitment of PBP. In contrast recruitment of PBP requires both an interaction with ER $\alpha$  and the presence of the p160 coactivator in the complex. The CoRNR box-substituted p160 factor is not only sufficient to promote the assembly of an active transcription complex, but was sufficient to reverse the effects of TAM and promote cell cycle progression in MCF-7 cells. This supports a pivotal role for p160 coactivators in estrogen signaling in breast cancer as suggested by the amplification of AIB1 in a subset of ER-positive breast cancers.

Based on our findings, we propose a dynamic model for the cyclic assembly of ER $\alpha$  transcription complexes (Figure 7). Rapidly, upon the addition of an agonist such as E2, liganded-ER $\alpha$  binds DNA. This is almost immediately followed by the recruitment of both a HAT-containing p160-p300 complex and the PBP complex. The p300 HAT complex modifies local chromatin structure through histone acetylation to facilitate RNA pol II recruitment. p300 acts in the initial cycle of transcription initiation, but not in subsequent cycles, perhaps suggesting that histone acetylation by p300 is long lived. Concurrent with the onset of transcription, the pol II C-terminal domain is phosphorylated and CBP replaces p300 in the complex bringing in pCAF. Subsequently, CBP acetylates p160 and leads to the release of p160

along with ER $\alpha$ . Finally, CBP and pCAF disassemble and the cycle is repeated. It is likely that cell-specific factors contribute to selective modulation of ER dynamics and cofactor sufficiency in important target tissues other than the breast, including the uterus, skeleton, brain, and cardiovascular system.

#### Experimental Procedures

##### Materials and Reagents

Antibodies used:  $\alpha$ ER $\alpha$ : Ab-1, Ab-3, and Ab-10 (NeoMarkers, Fremont, CA);  $\alpha$ AIB1 (affinity purified rabbit polyclonal serum),  $\alpha$ CBP (AC26) and  $\alpha$ p300 (RW128) (D. M. Livingston, Dana-Farber Cancer Institute, Boston, MA);  $\alpha$ pCAF (Y. Nakatani, Dana-Farber Cancer Institute, Boston, MA);  $\alpha$ RNA polymerase II: 8WG16 (J. B. Parvin, Brigham and Women's Hospital, Boston, MA) and H5 (Berkeley Antibody Company, Richmond, CA);  $\alpha$ Acetylated histone (Upstate Biotechnology, Inc., Lake Placid, NY);  $\alpha$ PBP (Atkins et al., 1999);  $\alpha$ SMRT (Guenther et al., 2000);  $\alpha$ N-CoR (Huang et al., 2000);  $\alpha$ VDR (Affinity BioReagents, Inc., Golden, CO).

##### ChIP

Cells were grown to 95% confluence in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% charcoal-dextran-stripped fetal bovine serum for at least 3 days. Following the addition of hormone for various times, cells were washed twice with PBS and cross-linked with 1% formaldehyde at room temperature for 10 min. Cells then were rinsed with ice-cold PBS twice and collected into 100 mM Tris-HCl (pH 9.4), 10 mM DTT and incubated for 15 min at 30°C and centrifuged for 5 min at 2000 g. Cells were washed sequentially with 1 ml of ice-cold PBS, buffer I (0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5), and buffer II (200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES, pH 6.5). Cells were then resuspended in 0.3 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, 1 $\times$  protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN) and sonicated three times for 10 s each at the maximum setting (Fisher Sonic Dismembrator, Model 300) followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by immunoclearing with 2  $\mu$ g sheared salmon sperm DNA, 20  $\mu$ l preimmune serum and protein A-sepharose (45  $\mu$ l of 50% slurry in 10 mM Tris-HCl, pH 8.1, 1 mM EDTA) for 2 hr at 4°C. Immunoprecipitation was performed for 6 hr or overnight at 4°C with specific antibodies. After immunoprecipitation, 45  $\mu$ l protein A-Sepharose and 2  $\mu$ g of salmon sperm DNA were added and the incubation was continued for another 1 hr. Precipitates were washed sequentially for 10 min each in TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 500 mM NaCl), and buffer III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1). Precipitates were then washed three times with TE buffer and extracted three times with 1% SDS, 0.1 M NaHCO<sub>3</sub>. Eluates were pooled and heated at 65°C for at least 6 hr to reverse the formaldehyde cross-linking. DNA fragments were purified with a QIAquick Spin Kit (Qiagen, CA). For PCR, 1  $\mu$ l from a 50  $\mu$ l DNA extraction and 21–25 cycles of amplification were used.

##### Nuclear Run-on

MCF-7 cells were grown in estrogen-depleted media for 3 days and treated with 100 nM of E2 for various times. Cell nuclei were isolated with NP-40 lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl<sub>2</sub>, and 0.5% [v/v] NP-40) and stored in liquid nitrogen in glycerol storage buffer (50 mM Tris.HCl, pH 8.3, 40% (v/v) glycerol, 5 mM MgCl<sub>2</sub>, and 0.1 mM EDTA). Nuclear run-on transcription was performed in the presence of 5  $\mu$ l of 10 mCi/ml [ $\alpha$ -<sup>32</sup>P]UTP. After treatment of the reaction with RNase-free DNase I and proteinase K, RNA was extracted with phenol/chloroform/isoamyl alcohol and hybridized to c-Myc and pS2 cDNAs, cathepsin D gene exon 1 DNA and  $\beta$ -actin oligo probe (Oncogene Research Products, Cambridge, MA) immobilized on a nylon membrane.

#### ChIP Re-IP and Real-time PCR

Complexes were eluted from the primary immunoprecipitation by incubation with 10 mM DTT at 37°C for 30 min and diluted 1:50 in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1) followed by reimmunoprecipitation with the second antibodies. ChIP Re-IPs of supernatants were done essentially as were the primary IPs. For real-time PCR detection of cathepsin D promoter, the probe and primers were: 6FAM-CCAAGGTTAAATTCAAAGTCCCAGC-TAMRA (probe); TCCAGACATCCTCTCTGGAA (forward primer); GGAGCGGAGGGTCCATTC (reverse primer). The ABI PRISM 7700 Sequence Detector and TaqMan 1000 Rxn PCR Core Reagents (Perkin Elmer, Branchburg, NJ) and sixty cycles of amplification were used.

#### Construction of CoRRN Box-Containing GRIP1 and PBP

CoRRN box-containing GRIP1 (subGRIP1) and PBP (subPBP) were constructed by standard molecular techniques. Each of the three NR boxes in GRIP1 was changed to the CoRRN box sequence -LEDIRKALMGSGFD- and both PBP NR boxes were changed to the chimeric CoRRN box sequence -HRLITLADHIEDIRKALMG-.

#### Flow Cytometry

MCF-7 cells were grown in phenol red-free DMEM supplemented with 10% charcoal-dextran-stripped fetal bovine serum for 24 hr and were cotransfected with pcDNA3-GFP and pCMX-GRIP1 or pcDNA3-subGRIP1. Forty-eight hours after the transfection, cells were treated with 100 nM E2 or 5  $\mu$ M TAM for another 16 hr. Cells were then collected and stained with propidium iodide using standard methods. Cell cycle data were collected with FACScan (Becton Dickinson Immunocytometry System) and analyzed with ModFit LT (Verity Software House, Inc., Topsham, ME).

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- The animals received water reward for fixating a projected light-emitting diode (LED), either on the wall in front of the monkey or on the rear-projection screen. Eye position was monitored using the magnetic search coil technique (37, 32). The room was illuminated by ~1000, small, incandescent lights (1.9 cd/m<sup>2</sup> on a black background) covering the three walls that were visible through the monkey's 90° × 90° field of view. The monkey faced the wall while the computer-controlled motorized sled either moved it along a circular path 127 cm in diameter, or positioned it at one of four stationary locations at 90° intervals on the circular path. All 63 neuronal responses were characterized by averaging across a pseudorandom sequence of four starting positions and six repetitions of each trial type. Each trial began with the illumination of the room-mounted light array, and the centered fixation point with recording started after 250 to 500 ms of centered fixation. Movement trials consisted of: 1 s of acceleration at 45°/s<sup>2</sup>, followed by 7.5 s of movement at 45°/s around 360° at a speed of 47 cm/s. The sled would then decelerate for 1 s at 45°/s<sup>2</sup>, the room light array was extinguished, and the monkey's reward was delivered. Stationary position trials required the monkey to maintain centered fixation in front of the room-mounted light array. After 8.5 s, the light array and fixation point were extinguished, and the reward was delivered. The position of the fixation point tracked sled movement so that the monkey maintained neutral, straight-ahead gaze throughout all trials.
- We recorded 107 neurons from four cerebral hemispheres in two rhesus monkeys. All studies presented are based on data sets that include neurons from both monkeys. All procedures were approved by the University of Rochester Committee on Animal Research and were consistent with Society for Neuroscience policy on the care and use of laboratory animals. Bilateral recording cylinders were placed over trephine holes in the parietal calvarium (stereotaxic coordinates: AP -2 mm, ML ± 15 mm, angle 0) above area MST. Microelectrode penetrations were made using epoxy-coated tungsten microelectrodes (FHC, Inc.) that were passed through transdural guide tubes into cortex (33). The location of recording sites on the anterior bank of the superior temporal sulcus was confirmed by magnetic resonance imaging with selected electrodes in place. MSTd neurons were identified by physiologic criteria: large receptive fields (>20° × 20°), which included the fovea with direction-selective responses that prefer large moving patterns rather than moving bars or spots (7, 2, 34). Single neuron discharges were isolated by using a dual-window discriminator and stored through the REX experimental control system (35).
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- In optic flow video simulation experiments, the monkey viewed a 90° by 90° rear-projection screen while maintaining neutral gaze by fixating on a red LED image at the center of the screen. Randomly interleaved trials consisted of computer-generated optic flow simulations, translational sled movement, or both. The optic flow video displays averaged ~1000 white dots (2.6 cd/m<sup>2</sup>) on a black background moving to simulate the visual motion pattern seen during observer movement in front of a stationary array of dots. The distance cue in the video simulation was either dot density or motion parallax (24). Accompanying translational movement matched the direction and speed of the optic flow video simulations and was identical to CW and CC circular translational movement presented when the monkey viewed the room-mounted lights. All 44 neuronal studies included a pseudorandom sequence of six repetitions of each stimulus type.
- Supplementary figures and details of video stimuli are available on Science Online at [www.sciencemag.org/cgi/content/full/295/5564/2462/DC1](http://www.sciencemag.org/cgi/content/full/295/5564/2462/DC1)
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## Molecular Determinants for the Tissue Specificity of SERMs

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Selective estrogen receptor modulators (SERMs) mimic estrogen action in certain tissues while opposing it in others. The therapeutic effectiveness of SERMs such as tamoxifen and raloxifene in breast cancer depends on their antiestrogenic activity. In the uterus, however, tamoxifen is estrogenic. Here, we show that both tamoxifen and raloxifene induce the recruitment of corepressors to target gene promoters in mammary cells. In endometrial cells, tamoxifen, but not raloxifene, acts like estrogen by stimulating the recruitment of coactivators to a subset of genes. The estrogen-like activity of tamoxifen in the uterus requires a high level of steroid receptor coactivator 1 (SRC-1) expression. Thus cell type- and promoter-specific differences in coregulator recruitment determine the cellular response to SERMs.

Tamoxifen and raloxifene are selective estrogen receptor modulators (SERMs) that bind the estrogen receptor (ER) and modulate ER-

mediated gene transcription. Tamoxifen is an effective treatment for all stages of hormone-responsive breast cancer and can prevent breast cancer in high-risk women (1). However, tamoxifen has partial estrogenic activity in the uterus and is associated with an increased incidence of endometrial hyperplasia and cancer. Raloxifene, approved for the prevention and treatment of osteoporosis in postmenopausal women, also appears to prevent

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breast cancer, but it does not increase the incidence of endometrial cancer. The National Cancer Institute supported "Study of Tamoxifen and Raloxifene" (STAR Trial) is currently being conducted to compare the safety and effectiveness of these two agents for the prevention of breast cancer in postmenopausal women (2).

The molecular mechanism underlying the tissue-specificity of SERM action is not clear. The crystal structures of the liganded ER hormone-binding domain (HBD) indicate that both tamoxifen and raloxifene can act as ER antagonists by competing with estradiol (E2) for binding and by inducing conformational changes that block the interaction of ER with coactivator proteins (3, 4). However, this does not explain how SERMs act as agonists or the differences in the spectrum of activity among various SERMs.

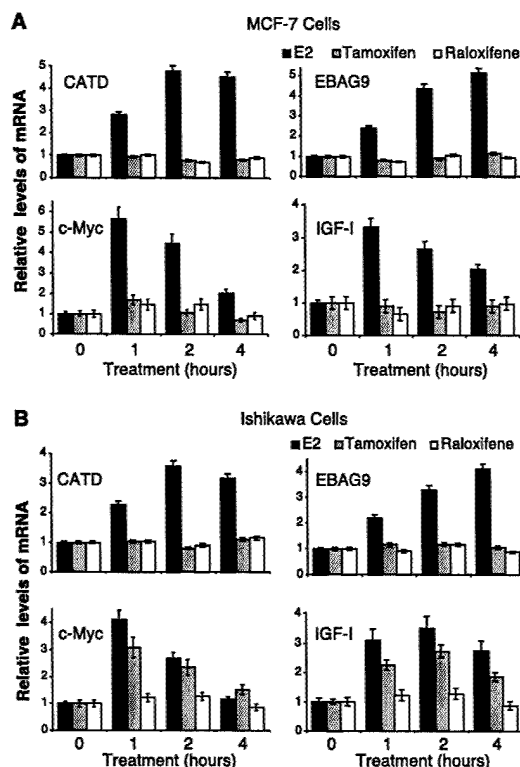
Estrogen receptor can regulate gene transcription either by binding directly to the promoter of target genes or by binding indirectly through a mechanism involving other transcription factors such as Sp1 and AP1. Genes regulated through direct ER binding, such as *CATD* (encoding cathepsin D) (5) and *EBAG9* (encoding ER-binding fragment-associated antigen 9) (6, 7), typically harbor an estrogen responsive element (ERE) with a consensus sequence of 5'-GGTCAnnnTGACC-3' in their promoters. Genes regulated by binding ER indirectly include *c-Myc* (8) and *insulin-like growth factor-1* (*IGF-1*) (9), whose promoters do not contain a classical ERE.

We examined transcriptional responses to tamoxifen and raloxifene in the mammary carcinoma cell line MCF-7 and the endometrial carcinoma cell line Ishikawa. In both cell types, estradiol (E2) induced the expression of both the directly bound ER target genes *CATD* and *EBAG9* in either MCF-7 or Ishikawa cells (Fig. 1). It is noteworthy, however, that in Ishikawa cells, but not in MCF-7 cells, tamoxifen, but not raloxifene, induced the expression of *c-Myc* and *IGF-1*, whose promoters do not contain a classical ERE. Similar tissue-specific results were also obtained in another endometrial carcinoma cell line ECC-1 and another mammary carcinoma cell line T47-D (10). These observations suggest that promoter context is one of the determinants for tissue-specific activities of tamoxifen.

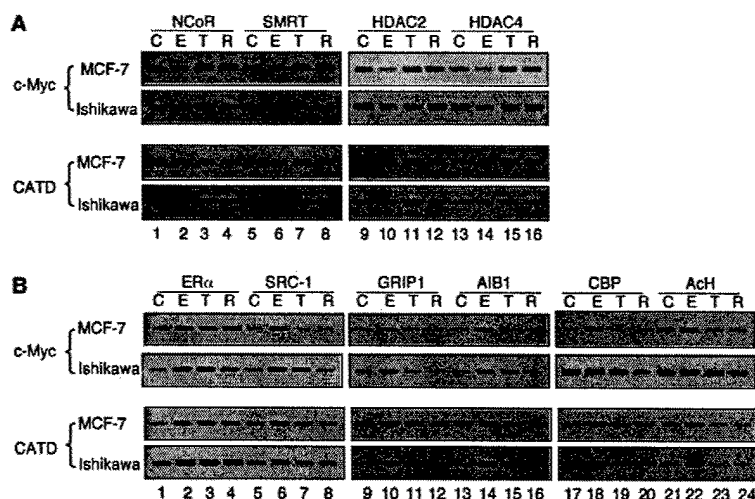
Estrogen receptor-mediated transcriptional activation is associated with the recruitment of coactivators, such as AIB1, GRIP1, SRC-1, CBP, p300, and pCAF, and subsequent histone acetylation (11-14). In contrast, antagonist-liganded ER is able to recruit corepressors (15-18). Previously, we showed in MCF-7 breast cancer cells that, when bound by tamoxifen, ER recruits the core-

pressors NCoR and SMRT and a subset of histone deacetylases (HDACs) to target promoters (18). Further examination of the recruitment of ER coregulators to target gene promoters by chromatin immunoprecipitation (ChIP) revealed that, in MCF-7 cells as well

as in Ishikawa cells, both tamoxifen and raloxifene induce the recruitment of corepressors and HDACs to the *CATD* promoter (Fig. 2A, lower panels). In striking contrast, in Ishikawa cells, but not in MCF-7 cells, instead of inducing the recruitment of a core-



**Fig. 1.** Stimulation of *c-Myc* and *IGF-1* expression by tamoxifen only in endometrial carcinoma cells. MCF-7 cells (A) or Ishikawa cells (B) were grown in phenol red-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% charcoal-dextran-stripped fetal bovine serum (FBS) for at least 3 days and left untreated or treated with 100 nM of 17 $\beta$ -estradiol (E2), 1  $\mu$ M of 4-hydroxytamoxifen (tamoxifen), or 1  $\mu$ M of raloxifene for different times. Total RNAs were extracted using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA), and the expression of *c-Myc*, *IGF-1*, *EBAG9*, or *cathepsin D* genes was measured by real-time reverse transcriptase (RT) polymerase chain reaction (PCR) using the ABI PRISM 7700 Sequence Detector and the TaqMan EZ RT-PCR kit (Applied Biosystems, Foster City, CA).



**Fig. 2.** Coregulator recruitment on ER target gene promoters. MCF-7 cells or Ishikawa cells were grown in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS for at least 3 days and left untreated (C) or treated with 100 nM of E2 (E), 1  $\mu$ M of 4-hydroxytamoxifen (T), or 1  $\mu$ M of raloxifene (R) for 45 min. ChIP assays (18) were performed using specific antibodies against (A) NCoR, SMRT, and HDAC2; and HDAC4 (Santa Cruz Biotechnology, Santa Cruz, CA); and (B) ER $\alpha$  (Ab-10, NeoMarkers, Fremont, CA); SRC-1 (a mouse monoclonal); GRIP1 (rabbit polyclonal); AIB1 (affinity-purified rabbit polyclonal); CBP (mouse monoclonal AC26); and acetylated histones (AcH) (Upstate Biotechnology, Lake Placid, NY).

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pressor complex, tamoxifen, but not raloxifene, induced the recruitment of a coactivator complex including SRC-1, AIB1, and CBP to the *c-Myc* promoter (Fig. 2B, upper panels, lanes 7, 15, 19). Tamoxifen-stimulated coactivator recruitment was accompanied by histone acetylation (Fig. 2B, upper panels, lane 23) consistent with the current model of gene activation by nuclear receptors. Tamoxifen-induced coactivator recruitment to the *c-Myc* promoter was also detected in ECC-1 cells and to the *IGF-I* promoter in both endometrial cancer cell lines (10).

As ER regulates the rate of gene transcription through its association with coregulators, the overall balance of the relative expression levels of coactivators and corepressors may be an important determinant of the tissue-specificity of SERMs. Examination of the expression levels of ER $\alpha$  and a variety of coregulators indicated similar levels of expression in MCF-7 and Ishikawa cells with the exception of SRC-1 (Fig. 3A), whose expression is low in MCF-7 compared with that in Ishikawa cells. The high level of SRC-1 expression in endometrial cells as compared with mammary cells was confirmed in several different cell lines (10). To investigate whether this difference in the level of

SRC-1 expression explained the ability of tamoxifen to stimulate *c-Myc* and *IGF-I* transcription, we first overexpressed SRC-1 in MCF-7 cells. Remarkably, expression of both *c-Myc* and *IGF-I* was stimulated by tamoxifen in SRC-1-transfected MCF-7 cells but not in GRIP1- or AIB1-transfected cells (Fig. 3B). This finding supports our conclusion that a high level of SRC-1 expression is sufficient to support the agonist activity of tamoxifen.

To determine whether SRC-1 is required for tamoxifen agonism, we silenced its expression in Ishikawa cells by RNA interference using short interfering RNA (siRNA) molecules (10, 19). Reduction of SRC-1 levels in Ishikawa cells eliminated tamoxifen-stimulated expression of *c-Myc* and *IGF-I* (Fig. 4A). It was interesting that SRC-1 silencing had only minimal effects on the E2-stimulated expression of *c-Myc* and *IGF-I*. In contrast, silencing of AIB1 expression led to a modest decrease in both E2- and tamoxifen-stimulated expression of *c-Myc* and *IGF-I* (Fig. 4A). These results strongly suggest that, although AIB1 plays a role in the maximal activity of both estrogen and tamoxifen, SRC-1 is specifically necessary for the agonist activity of tamoxifen in endometrial cells. These observations also suggest that the

specific coactivator requirements for estrogen- and tamoxifen-stimulated gene expression are distinct.

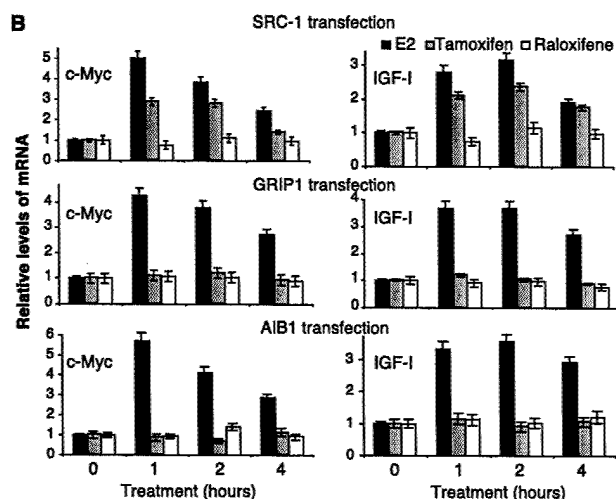
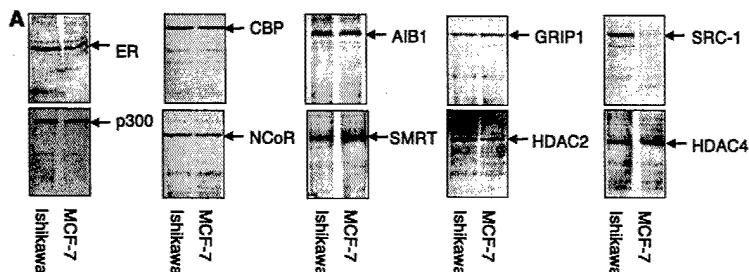
To determine whether SRC-1 expression was required for the growth stimulatory effects of tamoxifen in endometrial cells, we examined the effects of SRC-1 silencing on tamoxifen-stimulated cell-cycle progression in Ishikawa cells (Fig. 4B). As was the case for *c-Myc* and *IGF-I* expression, SRC-1 silencing abolished tamoxifen-stimulated cell-cycle progression but had only minimal effects on E2-stimulated cell-cycle progression. These results indicate that SRC-1 is a necessary determinant for the estrogenic effect of tamoxifen in endometrial cells.

In summary, in the breast where tamoxifen and raloxifene are both antagonists, both SERMs induce the recruitment of corepressors and not coactivators to ER target promoters. In contrast, in the endometrium where tamoxifen acts as an agonist and raloxifene as an antagonist, tamoxifen recruits coactivators instead of corepressors to ER target genes that do not contain a classical ERE, such as *c-Myc* and *IGF-I*. Finally, SRC-1 is required for the estrogen-like properties of tamoxifen in the endometrium.

It is unclear how coactivators are recruited by tamoxifen-bound ER to promoters that do not contain an ERE. Whether the ER AF-1 domain implicated in the agonist activity of tamoxifen (20–23) or the reported *in vitro* interactions of SRC-1 with AF-1 (24, 25) are relevant to the recruitment of SRC-1 by tamoxifen-bound ER remains to be shown. It may be that the binding of coactivators to tamoxifen-liganded ER is blocked when ER is directly bound to DNA through a classical ERE, but that when interacting with promoters indirectly, tamoxifen-bound ER adopts a conformation that promotes SRC-1 binding.

These experiments are based on a limited number of ER target genes and coactivators. It remains to be determined if *c-Myc* and/or *IGF-I* are the critical genes involved in tamoxifen-stimulated endometrial growth or endometrial cancer. However, *c-Myc* has been implicated in cell growth, proliferation, apoptosis, and malignant transformation (26). In addition, overexpression of *c-Myc* and *c-Myc* gene amplification have been reported in a variety of malignancies including endometrial cancer (27, 28). Likewise, the roles of *IGF-I* in cell proliferation and survival have also been well established (29).

Finally, our results do not exclude the possibility that other as-yet-undetermined cell-specific factors may contribute to the spectrum of SERM action. Our findings, however, do establish that cell type- and promoter-specific differences in coregulator recruitment plays a critical role in determining SERM function in the breast and uterus and offers a paradigm for understanding SERM action in other important target organs such as the brain, skeleton, and cardiovascular system.

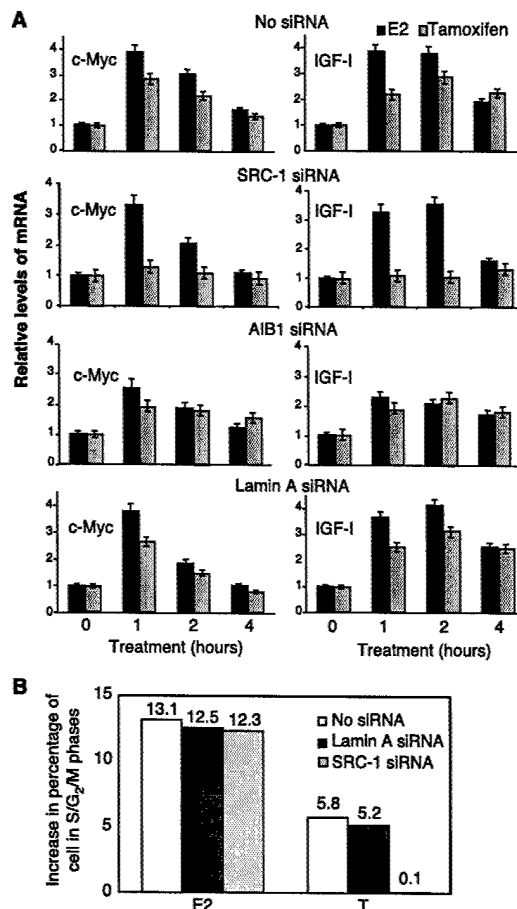


**Fig. 3. (A)** Comparison of SRC-1 expression levels in endometrial carcinoma cells and in mammary carcinoma cells. (A) Cells were grown in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS. Total proteins were extracted, and Western blottings were performed using antibodies against ER $\alpha$ , CBP, AIB1, GRIP1, SRC-1, p300 (mouse monoclonal RW128), NCoR, SMRT, HDAC2, and HDAC4. (B) Stimulation of *c-Myc* expression by tamoxifen in MCF-7 cells overexpressing SRC-1. MCF-7 cells were seeded in phenol red-free DMEM supplemented

with 5% charcoal-dextran-stripped FBS for 24 hours and were transfected with an expression construct for SRC-1, GRIP1, or AIB1 by using the Lipofectamine 2000 Reagent (Invitrogen Corp.). Forty-eight hours after transfection, cells were treated with 100 nM of 17 $\beta$ -estradiol (E2), 1  $\mu$ M of 4-hydroxytamoxifen (tamoxifen), or 1  $\mu$ M of raloxifene for different times. The Trizol Reagent was used to extract total RNAs for measuring mRNA level by real-time RT-PCR.

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**Fig. 4. (A)** The effect of SRC-1 silencing on tamoxifen-stimulated gene expression in Ishikawa cells. Ishikawa cells were seeded into 10-cm polystyrene cell-culture dishes (Becton Dickinson, Franklin Lakes, NJ) with phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS for 24 hours and transfected with 5  $\mu$ g/dish of double-stranded, short interfering RNAs (siRNAs) for SRC-1, AIB1, or lamin A/C using the Oligofectamine Reagent (Invitrogen Corp.). Single-stranded RNAs were synthesized by Dharmacon Research, (Lafayette, CO). Before transfection, single-stranded RNAs were incubated at 90°C for 1 min, followed by annealing in annealing buffer (100 mM potassium acetate, 30 mM Hepes-KOH, pH 7.4, and 2 mM magnesium acetate) at 37°C for 2 hours. Forty-eight hours after transfection, cells were treated with 100 nM of 17 $\beta$ -estradiol (E2), 1  $\mu$ M of 4-hydroxytamoxifen (Tamoxifen), or 1  $\mu$ M of raloxifene. The TRizol reagent was used to extract total RNAs for analyzing c-Myc and IGF-1 mRNA by real-time RT-PCR. Transfection efficiency was monitored by cotransfection with an *Escherichia coli lacZ* construct. **(B)** The effect of SRC-1 silencing on tamoxifen-stimulated cell-cycle entry. Ishikawa cells grown in phenol red-free DMEM supplemented with 5% charcoal-dextran-stripped FBS were cotransfected with 5  $\mu$ g of SRC-1 siRNAs and a green fluorescent protein construct (pEGFP, Clontech) or cotransfected with lamin A siRNA and pEGFP. Forty-eight hours after transfection, cells were treated with 100 nM of 17 $\beta$ -estradiol (E2) or 1  $\mu$ M of 4-hydroxytamoxifen (T) for another 16 hours. Cells were then collected and resuspended in PBS with 2% glucose and 3% paraformaldehyde. After permeabilization with ethanol, cells were stained with propidium iodide solution (69  $\mu$ M propidium iodide and 38 mM sodium citrate). Cell-cycle data were collected with FACScan (Becton Dickinson Immunocytometry System) and analyzed with ModFit LT (Verity Software House, Topsham, ME).



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## Television Viewing and Aggressive Behavior During Adolescence and Adulthood

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Television viewing and aggressive behavior were assessed over a 17-year interval in a community sample of 707 individuals. There was a significant association between the amount of time spent watching television during adolescence and early adulthood and the likelihood of subsequent aggressive acts against others. This association remained significant after previous aggressive behavior, childhood neglect, family income, neighborhood violence, parental education, and psychiatric disorders were controlled statistically.

Three to five violent acts are depicted in an average hour of prime-time television and 20 to 25 violent acts are depicted in an average hour of children's television (1-3).

Research has indicated that viewing television violence is associated with aggressive behavior (4-6). However, important questions regarding the nature and direction of

this association remain unanswered. Several theories hypothesize that television violence contributes to the development of aggressive behavior (7, 8). An alternative hypothesis is that some or all of the association is due to a preference for violent television programs among aggressive individuals (9). Research has provided support for both hypotheses (10). It has also been hypothesized that certain environmental characteristics, such as living in an unsafe neighborhood and being raised by neglectful parents increase the likelihood of both aggressive behavior and viewing televised violence. This hypothesis has not been extensively investigated.

Experimental and longitudinal studies have provided considerable support for the hypothesis that children's viewing of televised violence is associated with subsequent increases in aggressive behavior (11). However, most of these studies have inves-