Recruitment of Distinct Chromatin-modifying Complexes by Tamoxifen-complexed Estrogen Receptor at Natural Target Gene Promoters in Vivo*

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Tamoxifen, a breast cancer therapeutic, is a tissue-selective estrogen receptor modulator (SERM), which acts as an antiestrogen in the mammary tissue and displays estrogenic activity in other tissues such as bone and uterus. In order to understand the mechanisms underlying the antiestrogenic effect of this prototype SERM, we performed an analysis of the cofactors that interact with ER complexed with 4-hydroxytamoxifen (OHT) at natural target genes in a human breast tumor cell line MCF-7. Employing chromatin immunoprecipitation (ChIP), we observed that treatment with OHT rapidly induces the binding of ERα to the E-responsive promoter regions of pS2 and c-myc genes. Promoter-bound OHT-complexed ERα coordinately recruited the components of a multiprotein complex containing the corepressor NCoR, histone deacetylase 3 (HDAC3), and a WD40-repeat protein TBL1. Surprisingly, the OHT-complexed ERα also recruited a chromatin-remodeling NuRD complex in which histone deacetylase 1 (HDAC1) is associated with several polypeptides including metastasis-associated protein 1/2 (MTA1/2), and SWI2/SNF2-related ATPase Mi2. Kinetic studies revealed that following OHT addition the recruitment of these HDAC complexes to pS2 or the c-myc promoter occurs in a sequential manner; the NCoR-HDAC3 complex is recruited earlier than the NuRD complex. Serial ChIP experiments indicated that the ER-NCoR-HDAC3 and ER-NuRD complexes are distinct, and they do not occupy the target gene promoter simultaneously. We also established a close temporal link between the appearance of the HDAC complexes at the E-responsive regions of pS2 and c-myc promoters, local hypoacetylation of specific lysine residues in N-terminal tails of histones H3 and H4, and disappearance of RNA polymerase II from the target gene loci. Collectively, our studies indicated that transcriptional repression by tamoxifen-bound ER at E-regulated gene promoters involves a dynamic interplay of multiple distinct chromatin-modifying/remodeling complexes.

The steroid hormone estrogen (E) is a key regulator of growth and development of normal breast tissue (1, 2). This hormone also triggers metastatic activity of breast cancer cells by an unknown mechanism. In the target cell, estrogen exerts its effects by binding to intracellular E receptor subtypes, ERα and ERβ (3). The hormone-occupied E receptors (ER) bind to the estrogen-responsive DNA sequences in the genome and influence the expression of specific gene networks, which control cell proliferation and differentiation. ERα or ERβ displays a range of transcriptional activities, depending on whether the receptor is occupied by hormone or synthetic analogs known as selective estrogen receptor modulators (SERMs) (4–6). SERMs, such as tamoxifen and raloxifene, mimic estrogen action in certain tissues while opposing it in others. Tamoxifen behaves as an antiestrogen in the breast and is an effective treatment for hormone-responsive breast cancer (7, 8). In the uterus, however, tamoxifen is estrogenic (4–6). The mechanisms underlying the tissue-specific actions of SERMs are currently under investigation in several laboratories.

Recent studies revealed that distinct cellular coregulatory complexes modulate transcriptional activity of nuclear hormone receptors, including ER (3, 9, 10). Ligand-bound ER recruits multiple coactivator complexes to mediate gene activation (11, 12). Certain of these coactivator complexes contain histone acetyltransferase (HAT) and/ or methyltransferase activities, suggesting that they might covalently modify histones, leading to alterations in chromatin organization and structure (9, 10). The recruitment of a coactivator complex containing HAT activity by a promoter-bound steroid receptor is thought to lead to local hyperacetylation of lysine-rich tails of histones H3 and H4, which may decondense nucleosomes and facilitate the binding of transcription factors to the underlying DNA regulatory elements, leading to gene activation (9, 10). Previous in vitro and in vivo studies indicated that ER bound to tamoxifen interacts with the corepressor NCoR or SMRT, which are known to associate with histone deacetylases (HDACs) (13–17). These findings raised the possibility that recruitment of a corepressor complex by a promoter-bound tamoxifen-complexed ER may induce local hypoacetylation of H3 and H4 to create a repressive chromatin conformation, leading to gene repression. Little, however, is known about the nature of the corepressor complex recruited by tamoxifen-bound ER at an E-responsive promoter in vivo to suppress gene transcription.

In the present study, we embarked on an analysis of the nature of the corepressor complexes recruited by tamoxifen-complexed ER at the promoters of E-responsive genes, pS2 and

HAT, histone acetyltransferase; ChIP, chromatin immunoprecipitation assay; GST, glutathione S-transferase; HDAC, histone deacetylase; OHT, 4-hydroxytamoxifen; SERM, tissue-selective estrogen receptor modulator.

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1 The abbreviations used are: E, estrogen; ER, estrogen receptor;
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C-myc, in human breast cancer cells. Using chromatin immunoprecipitation (ChIP), we found that tamoxifen-bound ER sequentially recruits two distinct multiprotein complexes harboring histone deacetylase activities to the target promoter. Most importantly, during tamoxifen-induced suppression of ER-mediated gene transcription, the time course of recruitment of the HDAC complexes precisely coincided with that of deacetylation of histones H3 and H4 tails at the target promoters, providing critical support for the hypothesis that tamoxifen functions as an antagonist in the breast cells by inducing chromatin modification.

MATERIALS AND METHODS

Reagents—1β-Estradiol and progesterone were purchased from Sigma Chemical Co. 4-Hydroxytamoxifen was purchased from Tocris Cookson, Ellisville, MO.

Cell Culture—MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Before each experiment involving treatment with E or OHT, cells were grown for 3–4 days in phenol red-free Dulbecco's modified Eagle's medium supplemented with 5% dextran charcoal-stripped fetal bovine serum.

Chromatin Immunoprecipitation—Chromatin immunoprecipitation was performed essentially as described previously with minor modifications (16, 18). The estrogen receptor responsive regions of pS2 (~529 to ~262), and c-myc (~62 to +185) were amplified by PCR. The amplified products were analyzed by agarose gel electrophoresis using ethidium bromide staining and visualized in a KODAK Image Station 44FX.

Antibodies—Rabbit polyclonal antibodies against ERα, Sin3A, Mi2, MTA1, and TBL-1 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. The anti-HDAC1 antibody was raised against a synthetic peptide KEKEFPAKGVKEEKLKA corresponding to the C-terminal region 466–482 of human HDAC1 (Cell Signaling Technology, Beverly, MA). Rabbit anti-HDAC3 antibody was directed against a synthetic peptide NEFFYGDHDNKSDEVEI corresponding to the C-terminal region 411–428 of human HDAC3. Each antibody was specific for its antigen and did not show any cross-reactivity with other HDACs in Western blot experiments. Anti-acetyl histone H4, anti-acetyl histone H3 (Lys-9 and Lys-14), and anti-acetyl histone H3 (Lys-14) were purchased from Upstate USA, Inc., Charlottesville, VA. RNA polymerase II antibody was obtained from Covance, Berkeley, CA. Rabbit anti-NCoR antibody was raised against C-terminal amino acids 2057–2453 of murine NCoR fused to GST (15).

Serial ChIP—Following primary immunoprecipitation, the cross-linked complexes were eluted from the immunoprecipitate by incubation with an elution buffer (1% SDS, 50 mM NaHCO3) at room temperature for 30 min, and then diluted 1:20 in Re-IP dilution buffer (1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 10 mM TrisCl, pH 8.0, 140 mM NaCl, and 1 mM phenylmethylsulfonyl fluoro) followed by reimmunoprecipitation with a second set of antibodies.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)—Total RNA was isolated from MCF-7 cells treated with either 17β-estradiol or 4-hydroxytamoxifen using Trizol Reagent (Sigma). RNA (10 μg) was reverse-transcribed using ProSTAR™ First-Strand RT-PCR Kit (Stratagene). 10% of the RT product was amplified by PCR. The PCR fragments amplified were: 75–439 of pS2 mRNA, 965–1633 of c-Myc mRNA, and 509–789 of human acidic ribosomal phosphoprotein 36B4 mRNA.

RESEARCH

Treatment with OHT Induces Recruitment of ERα and NCoR to E-responsive Promoters—Tamoxifen behaves as an antagonist in the breast tissue (7). 4-Hydroxytamoxifen (OHT), an active cellular metabolite of tamoxifen, was used in this study. To explore the mechanisms underlying the inhibitory effects of OHT, we employed human MCF-7 breast cancer cells, which are enriched in ERα. We initially analyzed the effects of OHT on the expression of two previously characterized E-regulated genes, pS2 and c-Myc (19, 20). In the experiment described in Fig. 1, E or E plus OHT were added to MCF-7 cells grown in the absence of hormone, and the expression of pS2 and c-Myc mRNAs was monitored at different time points after ligand addition. Upon E (10−8 M) treatment, pS2 and c-Myc mRNAs were detected starting at 30 min (left, top, and middle panels).

The mRNA levels increased further at 45 min and remained elevated throughout the last time point at 60 min. In contrast, when OHT (10−8 M) was added together with E, neither pS2 nor c-Myc mRNA was detected at any time point (right, top, and middle panels). Treatment with OHT (10−7 M) alone did not induce pS2 or c-Myc mRNAs (data not shown). Addition of either E or OHT did not affect the expression of 36B4, a ribosomal protein gene used as a control (left and right, bottom panels). These results indicated that OHT effectively repressed E-induced expression of pS2 and c-Myc in MCF-7 cells.

We next determined whether the inhibitory effect of OHT on the pS2 and c-myc promoters is mediated through the recruitment of ER and the associated corepressors to the E-responsive regions of the pS2 and c-myc promoters (Fig. 2A). The pS2 promoter harbors an imperfect palindromic E-responsive element (at −393 to −405), which interacts directly with ER (19). The c-myc promoter, on the other hand, contains an E-responsive region (−25 to +141), which does not contain a classical ERE, but harbors a number of ERE half sites (20). We examined the interaction of ERα and NCoR with the E-responsive sites in these promoters by employing ChIP.

MCF7 cells were exposed to either E or OHT (10−7 M) for 40 min, and chromatins were isolated following chemical cross-linking. The cross-linked chromatins were fragmented and immunoprecipitated with antibodies against ERα and the corepressor NCoR. The DNA obtained from immunoprecipitated chromatin was amplified using primers flanking the E-responsive sites as well as primers obtained from unrelated regions several kilobases upstream of these sites (Fig. 2A). Our results indicated that in the absence of E or OHT, ERα did not interact with pS2 (Fig. 2B, upper panel, lane 4) or c-myc (Fig. 2C, upper panel, lane 7) promoter. In the presence of E or OHT, however, ERα exhibited binding to the region containing E-responsive sites of both pS2 (Fig. 2B, upper panel, lanes 5 and 6) and c-myc (Fig. 2C, upper panel, lanes 8 and 9) promoters. Interestingly, NCoR was recruited to the promoters in the presence of OHT, but not in the presence of E (Fig. 2B, upper panel, lanes 8 and 9; Fig. 2C, upper panel, lanes 11 and 12). Neither ERα nor NCoR showed any interaction with the distal promoter regions: ~3200 of pS2 or ~1800 of c-myc promoter (Fig. 2, B and C, lower panels), indicating that the observed receptor and cofactor interactions were restricted to the E-responsive promoter regions. Collectively, these results indicated that OHT-mediated repression of pS2 and c-myc genes involves interaction of ERα with the E-responsive promoter region, and the recruitment of the corepressor NCoR.

**Fig. 1. Regulation of pS2 and c-Myc mRNAs by E and OHT.** MCF-7 cells grown in E-free culture medium were treated with E (10−8 M) in the absence or presence of OHT (10−7 M) for indicated times. Cells were harvested at various time points, total RNA was isolated from these cells, and the expression of pS2, c-Myc, and ribosomal protein 36B4 mRNAs was analyzed by RT-PCR (28 cycles), using gene-specific primers.
MCF-7 cell were treated with vehicle (V) or E (10^{-8} M) that was reversed cross-linked and amplified by PCR (28 cycles). The input lanes represent soluble chromatin (1%). The PCR primers used in ChIP reactions to amplify promoter regions containing the E-responsive sites as well as unrelated upstream sequences are indicated by short bars. B, MCF-7 cell was treated with vehicle (V) or E (10^{-8} M) or OHT (10^{-7} M) for 45 min. The cells were then subjected to ChIP protocol. The immunoprecipitations were performed with antibodies against ERα (lanes 4–6) or NCoR (lanes 7–9) or normal rabbit IgG as a negative control (lanes 10–12). The isolated promoter DNA fragments were amplified by PCR using a pair of primers covering the ERE region (−529 to −262) of pS2 gene and another pair of primers covering the distal 5′-flanking region (−3368 to −3046) of pS2 gene (NCBI X05030 and ENSG00000160182). The input lanes represent soluble chromatin (1%) that was reversed cross-linked and amplified by PCR (28 cycles). V, E, and T represent treatments with vehicle, E, and OHT, respectively. Primers covering the ERE region (−529 to −262) of pS2 promoter and the ERE region (−62 to +185) of c-myc promoter were used.

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Fig. 3. Recruitment of HDAC-1 and HDAC-3 to pS2 and c-myc promoters in response to OHT. MCF-7 cells grown in E-free culture medium were treated with OHT (10^{-7} M) for 45 min and then subjected to ChIP protocol, using antibodies against normal rabbit IgG (upper panel, lanes 4–6), ERα (upper panel, lanes 7–9), NCoR (upper panel, lanes 10–12), HDAC-1 (upper panel, lanes 13–15; lower panel, lanes 1–3), HDAC-3 (upper panel, lanes 16–18; lower panel, lanes 4–6). V, E, and T represent treatments with vehicle, E, and OHT, respectively. Primers covering the ERE region (−529 to −262) of pS2 promoter and the ERE region (−62 to +185) of c-myc promoter were used.

Components of a NuRD Complex Are Also Recruited by OHT-ER—We next investigated the identity of the HDAC1 complex recruited by OHT-ER at the pS2 and c-myc promoters. Previous studies identified two distinct classes of protein complexes containing HDAC1 in mammalian cells: the Sin3 complex and the NuRD complex (30). Although these two complexes share the histone deacetylases HDAC1 and HDAC2, and the histone-lysine demethylase KDM2A, they also contain distinct polypeptides that are unique for each complex. The signature polypeptides of the NuRD complex are WD40-repeat protein TBL1 (27–29), and TBL1-associated polypeptide SAP 30, and SAP 18 (31). On the other hand, signature polypeptides that are unique for each complex. The signature polypeptides of the NuRD complex are WD40-repeat protein TBL1 (27–29), and TBL1-associated polypeptide SAP 30, and SAP 18 (31). In addition to HDAC1 and HDAC2, the NuRD complex contains additional polypeptides such as WD40-repeat protein TBL1 (27–29), and TBL1-associated polypeptide SAP 30, and SAP 18 (31). Recently, both HDAC1 and HDAC3 were recruited to these promoters in the presence of OHT (upper panel, lanes 15 and 18; lower panel, lanes 3 and 6).

OHT-ER recruits both HDAC 1 and HDAC3 to the Target Promoter—A rapidly growing body of evidence links corepressor recruitment by nuclear hormone receptors to the recruitment of HDACs during transcriptional repression (22–24). Previous studies initially reported that NCoR or SMRT is associated with a Sin3-HDAC1 complex (25, 26). Later on, other laboratories provided convincing evidence that cellular NCoR or SMRT associates with HDAC3 rather than HDAC1 (27–29). It is, therefore, important to analyze the identity of the NCoR-HDAC complex that is recruited by nuclear receptors at the target promoters.

We examined by ChIP analysis whether HDAC1 or HDAC3 or both are recruited along with NCoR to pS2 and c-myc promoters upon treatment of MCF-7 cells with OHT. The anti-HDAC1 and anti-HDAC3 antibodies used in this experiment were directed against the peptide epitopes corresponding to amino acids 466–482 of human HDAC1 and amino acids 411–428 of human HDAC3, respectively. These antibodies specifically recognized their cognate HDACs and did not show any cross-reactivity with other HDACs in Western blot experiments (data not shown). Neither HDAC1 nor HDAC3 was associated with pS2 or c-myc promoter in the absence of any ER ligand or in the presence of E (Fig. 3, upper panel, lanes 13 and 14, lanes 16 and 17; lower panel, lanes 1, 2, 4, and 5). Surprisingly, both HDAC1 and HDAC3 were recruited to these promoters in the presence of OHT (upper panel, lanes 15 and 18; lower panel, lanes 3 and 6).

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FIG. 4. Recruitment of the components of an NCoR-HDAC3 complex to pS2 and c-myc promoters by OHT-ER. MCF-7 cells were treated with vehicle (V) or OHT (10^{-7} M) for 45 min and subjected to ChIP using antibodies against NCoR (lanes 3 and 4), HDAC-3 (lanes 5 and 6), TBL1 (lanes 7 and 8), and normal rabbit IgG (lanes 9 and 10). The upper and lower panels describe factor recruitment to pS2 and c-myc promoters, respectively.

ATPase domain-containing Mi2 protein, metastasis-associated protein 1/2 (MTA1/2), and the methyl-CpG-binding domain-containing protein MBDD3 (32–35).

We performed ChIP to determine whether any of these two HDAC1-containing complexes is recruited by OHT-ER at the target promoter. As described in Fig. 5, we did not detect any recruitment of Sin3A at pS2 or c-myc promoters in response to OHT (lanes 7 and 8). Similarly, we failed to detect any recruitment of SAP30, another signature component of the Sin3 complex, to these promoters in response to OHT (data not shown). In contrast, we observed the recruitment of at least three signature polypeptides of the NuRD complex, HDAC1 (top panels, lane 4; bottom panels, lane 4), MTA1/2 (bottom panels, lane 6), and Mi2 (top panels, lane 6), to the target promoters. These results indicated that the HDAC1-containing complex recruited by OHT-ER is a NuRD complex rather than a Sin3 complex. It is pertinent to mention here that in a related ChIP analysis, the Sin3A and SAP30 antibodies were able to individually immunoprecipitate an ER-corepressor complex induced by a different SERM at the same promoters.2 The lack of detection of a Sin3 complex at pS2 or c-myc promoter in response to OHT was, therefore, not due to the inability of the Sin3A or SAP30 antibody to immunoprecipitate the cognate antigen in cross-linked chromatin.

Sequential Recruitment of the NCoR-HDAC3 and the NuRD Complexes at the Target Promoter—We next investigated the time course of recruitment of the NCoR-HDAC3 and the NuRD complexes to the pS2 and c-myc promoters. In this experiment, the recruitments of ERα and various components of the two HDAC complexes at the target promoters were examined over a 40-min time course following addition of OHT to the MCF-7 cells. The recruitment of ERα to the pS2 and c-myc promoters was detected as early as 5 min after OHT addition (Fig. 6A, lane 2; Fig. 6B, lane 2). The recruitment of NCoR, TBL1, and HDAC3 was seen within 10 min of OHT addition (Fig. 6A, lane D, A, lane 3; B, lane 3). The coordinate recruitment of all three signature subunits of the NCoR-HDAC3 complex at the target promoters is consistent with previous reports that NCoR, HDAC3, and TBL1 exist in a multiprotein complex in animal cells.

We also determined the time course of recruitment of two key components of the NuRD complex, HDAC1, and Mi2, at the pS2 and c-myc promoters. As shown in Fig. 7, A and B, recruitment of these proteins was detected at 5 and 10 min after OHT addition, although ERα recruitment was observed at these time points (lanes 2 and 3). The recruitment of HDAC1 and Mi2 to the promoters was seen starting at 20 min following OHT addition (lane 4), and their levels at the promoters increased further at 40 min (lane 5). The co-recruitment of at least two subunits of the NuRD complex to the promoters supported our view that a NuRD complex is brought to the promoter by OHT-ER.

The results of the time course experiments (Figs. 6 and 7) suggested that the NCoR-HDAC3 and the NuRD complexes are recruited by the promoter-bound OHT-ER in a sequential manner. Whereas the three components of the NCoR-HDAC3 complex were coordinately recruited at the pS2 and c-myc promoters at 10 min after OHT addition, the components of the NuRD complex were not detectable at this time. Both HDAC1 and Mi2 proteins appeared at the promoters at 20 min after OHT treat-

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We cannot rule out the possibility that kinetic differences in the recruitment of these cofactors at the promoters may arise due to differential precipitation efficiency of the antibodies used in the ChIP assay. This possibility is, however, remote given the orchestrated manner in which multiple components of the NCoR-HDAC3 complexes were detected at the target promoter using antibodies against individual components. The antibodies against Mi2 and HDAC1 also placed these two components of the NuRD complex simultaneously at the promoter at 20 min. These results are consistent with a sequential recruitment of the NCoR-HDAC3 and the NuRD complexes to the pS2 and c-myc promoters.

The ER-NCoR-HDAC3 and ER-NuRD Complexes Are Distinct and Do Not Occupy the Target Promoter Simultaneously—Although the results described above suggested that two distinct classes of HDAC complexes are recruited to the target promoters by ER-OHT, it is important to determine whether these complexes occupy the same or different promoters at any given time. To distinguish between these possibilities, we performed serial ChIP reactions. Following exposure of MCF-7 cells to OHT for 40 min, the first round of immunoprecipitation was carried out with a HDAC3 antibody. It should be noted that at this time point, ERα as well as the components of both NCoR-HDAC3 and NuRD complexes were present at the target promoters (Figs. 6 and 7). The immunoprecipitated cross-linked DNA-protein complexes were then isolated and subjected to re-immunoprecipitation using antibodies against ERα, NCoR, HDAC3, and HDAC1. As expected, the cross-linked DNA-protein complexes isolated by the first round of immunoprecipitation (by HDAC3) were re-immunoprecipitated with antibodies against individual components. The antibodies against Mi2 and HDAC1 also placed these two components of the NuRD complex simultaneously at the promoter at 20 min. These results are consistent with a sequential recruitment of the NCoR-HDAC3 and the NuRD complexes to the pS2 and c-myc promoters.

We further extended these findings by performing reciprocal experiments in which we used a HDAC1 antibody during the first round of immunoprecipitation, and re-immunoprecipitated the isolated complexes with antibodies against ERα and several signature components of the NCoR-HDAC3 and NuRD complexes. The results of this experiment are presented in Fig. 8B. While the HDAC1 complexes isolated after primary immunoprecipitation contained ERα in an OHT-dependent manner (top, ps2, and c-myc panels, lane 6), they did not contain NCoR (lane 8). Furthermore, consistent with its proposed identity as a NuRD complex, the HDAC1 complex contained MTA1/2 (bottom, ps2, and c-myc panels, lane 8), but was devoid of NCoR (lane 6). Collectively, the results of the serial ChIP experiments revealed that (i) the ER-NCoR-HDAC3 and ER-NuRD complexes are distinct from each other, and (ii) the promoters that bound to the ER-NuRD complex did not simultaneously bind the ER-NCoR-HDAC3 complex and vice versa. Therefore, at any given time, a promoter-bound OHT-ERα is associated with only one of the two distinct HDAC complexes, the NCoR-HDAC3 complex or the NuRD complex.

**Fig. 7.** Time course of recruitment of the NuRD complex to pS2 and c-myc promoters in response to OHT. MCF-7 cells were treated with OHT (10^-7 M) and promoter occupancy by ERα, HDAC1, and Mi2 was examined at different times by ChIP using antibodies against these factors. A single lysate preparation was made at each time point and aliquots were assayed for the recruitment of each of the three factors. A, time course of recruitment of ERα, HDAC1, and Mi2 to pS2 promoter. B, time course of recruitment of the same factors to c-myc promoter.

**Fig. 8.** Serial ChIP to examine whether ER-NCoR-HDAC3 complex and ER-NuRD complex assemble to the same promoters. MCF-7 cells were treated with vehicle or OHT (10^-7 M) for 40 min and subjected to the cross-linking agent as per the ChIP protocol. Soluble chromatin then was prepared, divided into two aliquots, and immunoprecipitated using anti-HDAC1 or anti-HDAC3 antibody. V and T represent treatments with vehicle and OHT, respectively. A, one aliquot was immunoprecipitated with an anti-HDAC3 antibody. The resulting immune complex was isolated, and the bound proteins were eluted as described under “Materials and Methods.” The eluate was reimmunoprecipitated with antibodies against ERα (lanes 3 and 4), HDAC1 (lanes 5 and 6), HDAC3 (lanes 7 and 8), NCoR (lanes 9 and 10). B, another aliquot was immunoprecipitated using an anti-HDAC1 antibody. The resulting immune complex was isolated and the bound proteins were eluted as described under “Materials and Methods.” The eluate was reimmunoprecipitated with antibodies against ERα (upper panels, lanes 5 and 6), HDAC-1 (upper and lower panels, lanes 3 and 4), HDAC-3 (upper panel, lanes 7 and 8), NCoR (lower panel, lanes 5 and 6), MTA1/2 (lower panel, lanes 7 and 8), and normal rabbit IgG (upper and lower panels, lanes 9 and 10).
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**Time Course of OHT-induced HDAC Recruitment Overlaps with That of Histone Deacetylation and Loss of RNA Polymerase II Occupancy at the Target Promoters—**Previous studies suggested that recruitment of HDACs by transcriptional repressors reduces acetylation of histones in nucleosomes and creates a repressive chromatin conformation, leading to gene repression (22, 23, 36). Based on the observation that OHT induces recruitment of HDAC1 and HDAC3 to E-responsive promoters, one would predict that these enzymes would induce promoter-restricted deacetylation of specific lysine residues in N-terminal tails of histones H3 and H4. To examine the link between HDAC recruitment and histone deacetylation during OHT-dependent repression of E-induced gene transcription, we designed an experiment in which we added OHT to MCF-7 cells pre-treated with E for 1 h. At different time points following OHT addition, we monitored pS2 and c-Myc mRNA levels by RT-PCR, assessed HDAC recruitment, and monitored acetylation status of histones H3 and H4 at the E-responsive regions of the target promoters using ChIP.

Prior to the addition of OHT, we noted a substantial E-induced accumulation of pS2 or c-Myc mRNAs in these cells (Fig. 9A, lane 1). As expected, addition of OHT, which blocks ER-mediated transcription, led to a progressive decline in the steady state level of each mRNA. The level of pS2 mRNA was markedly reduced by 45 min (top panel, lanes 6 and 7). A significant reduction in c-Myc mRNA level was noticeable at 45 min and further decline was seen at 60 min (middle panel, lanes 6 and 7). Control cells treated with E alone for 2 h maintained a high level of pS2 or c-Myc mRNA expression (lane C).

In ChIP assays, the recruitment of HDAC3 to either promoter was observed as early as 10 min following OHT addition and its promoter occupancy continued to increase with time (Fig. 9, B and C, panel a). The HDAC1 recruitment was first detected at 20 min and it also progressively increased with time (Fig. 9, B and C, panel b). This sequential recruitment of HDAC3 and HDAC1 is consistent with the results of earlier kinetic experiments (Figs. 6 and 7).

To analyze the acetylation states of histones H3 and H4, we performed ChIP assays using antibodies that specifically recognize the acetylated forms of these histones. While the anti-H3 antibody was raised against H3 acetylated at Lys-9 and Lys-14, the anti-H4 antibody was directed against H4 acetylated at multiple lysines: Lys-5, Lys-8, Lys-12, and Lys-16. Following a brief treatment of MCF-7 cells with E, we noted significant levels of H3 and H4 acetylation at the E-responsive promoter sites (Fig. 9, B and C, panels c and d, lane 1). The level of acetylated lysines of H3 at the pS2 promoter started to decline starting at 30 min following OHT addition (Fig. 9B, panel c, lane 5). The deacetylation progressed further at 45 min and was completed by 60 min (panel c, lanes 6 and 7). When we used an antibody directed specifically against Lys-14 of H3, we obtained essentially similar results (data not shown). The level of acetylated lysines of histone H4 at the promoter also declined at a similar rate (Fig. 9B, panel d). Substantial deacetylation of H4 lysines was evident at 45 min and it was complete by 60 min (panel d, lanes 6 and 7).

A similar pattern of OHT-induced deacetylation of H3 and H4 was observed at the c-myc promoter (Fig. 9C, panels c and d). In this case, however, the rate of deacetylation of H3 was slightly slower than that of H4. Overall, the kinetics of disappearance of acetylated H4 and H3 from the pS2 and c-myc promoters matched well with the kinetics of OHT-induced recruitment of the HDACs. The peak of HDAC recruitment (Fig. 9, B and C, panels a and b, lanes 6 and 7) clearly coincided with the peak of histone deacetylation activity (panels c and d, lanes 6 and 7). These results strongly support the view that the recruitment of the HDAC-containing complexes during OHT-induced repression of E-mediated transcription leads to hyperacetylation of histones H3 and H4 at the target promoter.

We further analyzed the link between histone deacetylation and transcriptional repression at the target promoters by examining the occupancy of pS2 and c-myc gene loci by the RNA polymerase II. For this purpose, we employed three sets of PCR primers for each gene: one spanning an upstream untranscribed region, one spanning the TATA box in the proximal promoter and the initial transcribed region, and one spanning the end of the transcribed region. As shown in Fig. 9, D and E, in the presence of E, RNA polymerase II was found to be associated with the region containing the TATA box and the sequences within exon 1 (panel b, lane 8) of either pS2 or c-myc gene. This enzyme was also present at the end of the transcribed region within exon 3 of each gene, consistent with active transcription of these genes in the presence of E (panel c, lane 8). As expected, we did not detect any association of RNA polymerase II with an upstream untranscribed region (panel a, lane 8). When OHT was added, the RNA polymerase II occupancy of the region containing the TATA box as well as the transcribed region containing exon 3 was not significantly altered during the first 30 min. The polymerase II occupancy of these regions, however, sharply decreased, starting at 45 min, and then fell to a barely detectable or undetectable level by 60 min (panels b and c, lanes 13 and 14). Strikingly, the kinetics of disappearance of RNA polymerase II from the proximal promoter and transcribed region is in excellent agreement with the kinetics of deacetylation of histones H3 and H4 as described in Fig. 9C. These results support the hypothesis that serial chromatin modifications and remodeling by the HDAC3 and HDAC1 complexes create a repressive chromatin that leads to a reduction of RNA polymerase II occupancy within the target gene and results in inhibition of transcription. To our knowledge, this report is the first clear evidence linking specific chromatin modifications to transcriptional repression at natural E-responsive gene promoters by an antagonist SERM.

**DISCUSSION**

Tamoxifen counteracts the proliferative effects of E in the breast and is currently the most widely used therapy for hormone-dependent breast cancer (7, 8). Approximately 30% of the breast cancer patients with metastatic tumors exhibit short-term remissions in response to therapy with tamoxifen (37). Unfortunately, the tumors develop resistance to this drug over time and escape tamoxifen inhibition while the therapy is ongoing. The tumors actually become tamoxifen-dependent, as withdrawal of the drug leads to temporary remission in tumor growth (37). The molecular basis of this paradoxical switch of tamoxifen from an antagonist to an agonist remains poorly understood. Clearly, in order to understand how cells become resistant to the antiestrogenic actions of tamoxifen, it is important to decipher the mechanistic steps that underlie the control of E-regulated genes by tamoxifen-complexed ER. As a first step toward that goal, we analyzed the cellular cofactors that are recruited by OHT-ER to repress transcription of well-defined E-responsive genes, pS2 and c-myc, in MCF-7 breast cancer cells. Our study revealed that OHT induces rapid recruitment of ERα as well as the components of two distinct chromatin-modifying complexes to these promoters. These results thus offer new insights into the molecules that participate in chromatin-based events underlying the antagonistic activity of tamoxifen.

Previous studies indicated that NCoR or SMRT exists in a multiprotein complex containing HDAC3 and additional polypeptides such as TBL1 and G protein suppressor 2 (27–29). Our present studies revealed that ER was recruited to pS2 and...
c-myc promoters within 5 min of OHT addition (Figs. 6 and 7). This was followed by the recruitments of NCoR, TBL1 and HDAC3 at 10 min. This pattern of recruitment is consistent with the concept that promoter-bound OHT-ER recruits a multiprotein NCoR-HDAC3 complex. This notion is further strengthened by our findings in the serial ChIP experiments (Fig. 8A) that immunoprecipitation of a cross-linked chromatin complex by an anti-HDAC3 antibody co-precipitates ERα, NCoR, and HDAC3.

Although HDAC3 is reported to be the most prevalent HDAC...
associated with NCoR in HeLa cells, we also observed the surprising recruitment of HDAC1 to the E-responsive promoters in response to OHT in MCF-7 cells. HDAC1 is known to exist in two major types of multiprotein complexes: the Sin 3 complex and the NuRD complex (30–34). Using antibodies against Sin3A and SAP30, two signature components of the Sin3 complex, we found no evidence that a Sin3 complex is recruited to pS2 or c-myc promoter in response to OHT treatment (Fig. 5 and data not shown). In contrast, two signature components of the NuRD complex, Mi2 and MTA1/2 were recruited to the target promoters in response to OHT. The time course of appearance of Mi2 precisely overlapped with that of HDAC1, consistent with the notion that an intact NuRD complex is brought to the promoters by OHT-ER. This view was further supported by serial ChIP experiments (Fig. 8B), which confirmed the existence of a multiprotein complex containing ERα, HDAC1, and MTA1/2 at the target promoters.

Interestingly, the NCoR-HDAC3 and NuRD complexes appeared at the pS2 or c-myc promoter in a sequential fashion following OHT addition. All three signature components of the NCoR-HDAC3 complex were recruited at the target promoter within 10 min. In contrast, no component of the NuRD complex occupied the promoter at this time. Two components of the NuRD complex, Mi2 and HDAC1, were co-recruited to the promoter at 20 min. At subsequent time points, both complexes were found to be associated with the target promoters. This observation raised the possibility that between 20–60 min after OHT addition, both NCoR-HDAC3 and NuRD complexes are simultaneously bound to the E-responsive region of the promoter. Our serial ChIP experiments, however, ruled this possibility out as the promoters that bound the ER-NCoR-HDAC3 complex did not retain the NuRD complex and vice versa. These findings suggested that beyond 20 min of OHT treatment, two distinct promoter populations exist: one bound to the ER-NCoR-HDAC3 complex and the other bound to the ER-NuRD complex. It is possible that a single promoter is able to associate with both complexes, but only one complex occupies the promoter at any given time. Since we did not detect any obvious recycling of the complexes off the promoters over a 60 min time course following addition of OHT to the cells, we assume that any exchange of complexes at the target promoter must occur at a very rapid rate and is beyond the resolution of the ChIP assay. Indeed, recent studies showed that two distinct coactivator complexes exchange very rapidly (within 2.5 min) at E-responsive promoters (12). Furthermore, studies with fluorescent protein-tagged steroid receptor and coactivators in live cells using stably integrated hormone response element arrays indicated that receptor-coactivator complexes undergo molecular exchange within seconds (38, 39). We, however, cannot rule out the possibility that the two HDAC complexes are recruited to distinct subsets of pS2 or c-myc promoters that are inherently different in some unknown way.

The molecular interactions between OHT-ER and the components of the NCoR-HDAC3 and NuRD complexes remain to be determined. Previous studies indicated that NCoR or SMRT is the primary anchor of a multicomponent HDAC3-containing complex to thyroid hormone receptor (TR) (25–29). It has been demonstrated that within the C-terminal receptor interaction domain of the corepressor, two CoRNR boxes containing 1/LXXI/VI signature motifs mediate interaction with unliganded TR (40, 41). Direct interaction of NCoR with OHT-ERα was observed in vitro (13–15). Recent studies, however, suggested that the interaction between NCoR and OHT-ERα may not be mediated by the CoRNR boxes (42). The structural motifs that mediate the interaction of NCoR with antagonist-complexed ER, therefore, remain to be identified.

How is the NuRD complex brought to the E-responsive promoter? Our serial ChIP experiments demonstrated that the ER-NuRD complex is devoid of NCoR (Fig. 8B), making it unlikely that the NuRD complex is recruited via this co-repressor. It is possible that the NuRD complex is recruited via interaction of a subunit of this complex with OHT-ER. An interaction between recombiant MTA1 and ERα in vitro has been reported (43). It is however, unclear whether MTA1 interacts with ER in the context of an intact NuRD complex or whether it interacts with OHT-complexed ER at all. An alternative mode of recruitment of the NuRD complex is through interaction with the components of the histone core or underlying promoter DNA sequences. Certain HDAC-containing complexes are known to interact with methylated DNA to induce chromatin remodeling (44). Recent studies reported the isolation of a large histone deacetylase complex containing all the known subunits of the NuRD complex and additional polypeptides, including MBD2, a methyl-CpG-binding protein (45). This raises the intriguing possibility that the NuRD complex is recruited via interaction of the MBD2 subunit with methyl-CpG islands at the target promoters. Although the methylation status of pS2 and c-myc promoters during tamoxifen treatment of MCF-7 cells is unknown, it is interesting to note that, in certain cancer cell lines, the expression of several nuclear receptor genes are repressed through CpG methylation of their gene promoters (46).

It is well documented that the N-terminal tails of histones H2B, H3, and H4 are subject to a wide array of chemical modifications, including acetylation, methylation, phosphorylation, and ubiquitination, in response to various cellular signals (47–49). Ample evidence has now accumulated that nuclear receptor-associated coactivators and corepressors play critical roles in modulating acetylation states of histones H3 and H4 in nucleosomes (22–24). It is thought that hyperacetylation of the lysine-rich tails of histones H3 and H4 promotes the HAT activity of the coactivators induces decondensation of nucleosomes and facilitates the binding of transcription factors to the promoter regulatory elements, leading to gene activation. Hypoacetylation of H3 and H4 by corepressor-associated HDACs, on the other hand, creates a condensed chromatin conformation, leading to gene repression. It is, therefore, postulated that recruitment of protein complexes containing HAT or HDAC by a sequence-specific transcription factor at a target promoter may determine the transcriptional outcome through alteration of chromatin structure. Consistent with this hypothesis, OHT-induced repression of transcriptional activity of ERα was accompanied by the recruitment of multi-protein complexes containing HDAC1 and HDAC3 by OHT-ERα. We demonstrated that the kinetics of recruitment of HDAC1 and HDAC3 by OHT-ERα closely overlaps with that of deacetylation of specific lysine residues of N-terminal tails of histones H3 and H4 in the E-responsive regions of pS2 or c-myc promoter. Furthermore, the time course of RNA polymerase II occupancy of the target genes indicated a close temporal relationship between histone deacetylation and the disappearance of the polymerase from the proximal promoter and the transcribed regions. These results strongly support the concept that promoter-bound antagonist-complexed ER represses gene expression by recruiting HDACs, which in turn induces local hypoacetylation of histones and remodeling of chromatin, and creates a repressive chromatin conformation that hinders interaction of RNA polymerase II with the target genes.

The functional significance of the recruitment of two distinct HDAC-containing complexes by OHT-ER is presently unclear. It is possible that these complexes work together in a sequential manner to effect chromatin modifications that are essential...
for transcriptional repression. Recent studies provide support for modification-induced recruitment of chromatin-associated proteins to acetylated and methylated histone N termini (50, 51). It has been reported that deacetylation of Lys-9 of histone H3 by a HDAC allows methylation of this residue by a methyltransferase, thus triggering sequential events leading to repression. The Mi2 subunit of the NuRD complex harbors two chromodomains, which can potentially recognize dimethyl histone tails (51). It is, therefore, conceivable that recruitment of NCoR-HDAC3 and accompanying histone deacetylation permits subsequent methylation by a methyltransferase, and this in turn provides modification marks on the histone tails that serve as docking sites for the NuRD complex. In this model, the HDAC- and ATP-dependent nucleosome remodeling activities in the NuRD complex cooperate functionally to induce the changes that are necessary for transition of chromatin from a transcriptionally active to a transcriptionally silent state. Future studies will address the interdependence and precise functional roles of the multiple chromatin-modifying complexes involved in tamoxifen-induced gene repression.

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