Estrogens, acting through their nuclear receptors have a broad impact on target cells, eliciting a transcriptional response program that involves gene repression as well as gene stimulation. While much is known about the mechanisms by which the estrogen-occupied estrogen receptor (ER) stimulates gene expression, the molecular events that lead to gene repression by the hormone-ER complex are largely unknown. Because estradiol represses expression of the cyclin G2 gene, which encodes a negative regulator of the cell cycle, our aim was to understand the mechanism by which cyclin G2 is repressed by estrogen. We show that cyclin G2 is a primary ER target gene in MCF-7 breast cancer cells that is rapidly and robustly down-regulated by estrogen. Promoter analysis reveals a responsive region containing a half-estrogen response element and GC-rich region that interact with ER and Sp1 proteins. Mutation of the half-ERE abrogates hormone-mediated repression. Mutational mapping of receptor reveals a requirement for its N-terminal region and DNA binding domain to support cyclin G2 repression. Following estradiol treatment of cells, chromatin immunoprecipitation analyses reveal recruitment of ER to the cyclin G2 regulatory region, dismissal of RNA polymerase II, and recruitment of a complex containing N-CoR and histone deacetylases, leading to a hypoacetylated chromatin state. Our study provides evidence for a mechanism by which the estrogen-occupied ER is able to actively repress gene expression in vivo and indicates a role for nuclear receptor corepressors and associated histone deacetylase activity in mediating negative gene regulation by this hormone-occupied nuclear receptor.

In target cells, estrogen hormones act via their nuclear receptors to elicit a diverse transcriptional response program that involves gene repression as well as gene stimulation (1–3). Estrogen receptors (ERs)\(^3\) share their modular domain structure with the other members of the steroid and nuclear receptor superfamily and are comprised of an N-terminal domain characterized by a ligand-independent activation function (AF-1), a central DNA binding domain (DBD) followed by a hinge region, and a C-terminal ligand binding domain (LBD), which contains a ligand-dependent activation function (AF-2) important for coregulator recruitment (4, 5).

In the mammalian gland, the actions of estrogens are essential for normal growth and development. In breast cancer, the presence of ERs is associated with likely response to endocrine therapy, most usually treatment with antiestrogens such as tamoxifen, or estrogen depletion by the use of aromatase inhibitors (6). Estrogens enhance the proliferation of ER-positive breast cancer cells, and recent studies have shown that this enhancement of proliferation involves both stimulation of the expression of many genes associated with cell cycle progression, as well as the suppression of genes that block the cell cycle (7). Most previous studies, focusing on the mechanisms that lead to stimulation of gene expression, have demonstrated that ERs can directly bind to perfect or imperfect palindromic DNA estrogen response elements (EREs) (8) where they function as nucleation sites for assembly of the basal transcriptional machinery and a cohort of coactivators and chromatin remodeling complexes (e.g. p160 family, CBP/p300, mediator, and others) (9–11). ERs are also able to activate transcription through half-ERE sites (12, 13), as well as by tethering mechanisms that involve protein-protein interaction of ER with other DNA-bound transcription factors (i.e. Sp1, AP-1, NfκB) (14–16).

On the other hand, the molecular processes that lead to gene repression by the agonist ligand-occupied ER are largely unknown. Gene expression profiling on breast cancer cells using cDNA microarrays, which has proved to be a powerful tool for identifying estrogen receptor target genes, has revealed that in ER-containing breast cancer cells estrogen is able to down-regulate as many genes as it up-regulates (7, 17–19). Intriguingly, these down-regulated genes include cell cycle inhibitory genes, such as cyclin G2, and proapoptotic genes whose suppression by estradiol would enhance tumor cell growth and survival (7). The ability of estrogen to decrease cellular levels of cyclin G2, a negative regulator of the cell cycle, could play a critical role in the ability of this hormone to accelerate the transition of breast cancer cells through the cell cycle.

Cyclin G2 shares homology with a recently discovered subgroup of cyclins that includes cyclin G1 (53% amino acid sequence identity) and cyclin I (30% amino acid sequence identity). These cyclins are expressed mainly in highly differentiated tissues, such as heart, muscle, and various regions of the brain (20–22). This group of cyclins has an atypical function, which does not seem to be associated with cell cycle progression but, instead, involves negatively controlling the cell cycle and maintaining cells in a quiescent state (20).

Because we have observed that cyclin G2 is robustly down-regulated following estrogen exposure of breast cancer cells, our aim in this study has been to elucidate the mechanism by which agonist hormone-occup-
plied ER is able to elicit gene repression. Investigation of the cyclin G2 gene promoter, mapping of ER regions involved in cyclin G2 repression, and chromatin immunoprecipitation and other approaches provide evidence for an active mechanism by which the estrogen-bound ER is able to negatively regulate expression of this gene by the recruitment of nuclear receptor corepressors and associated HDACs. The ability of the estrogen-occupied ER to utilize distinct molecular strategies for gene repression versus gene activation appears to underlie the versatility of this transcription factor in enacting ligand-mediated gene expression in a promoter- and gene-specific manner. Such versatility of function underlies the integrated pattern of transcriptional response that breast cancer cells have to hormone treatment.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Treatments, and RNA Extraction**—MCF-7 and HeLa cells were routinely cultured in minimal essential medium (Sigma) supplemented with 5% heat-inactivated calf serum (Hyclone, Logan, UT) and 1% antibiotics. Before treatments or transfections, the cells were maintained in phenol red-free minimal essential medium containing 5% charcoal-stripped calf serum for a minimum of 4 days with the medium changed every other day. Cells were generally treated with 10 nM E2 alone or in combination with other ligands for 2 h (unless different concentrations and times are indicated in figure legends). Total RNA was harvested and prepared using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. Gliotoxin was purchased from Sigma.

**Quantitative Real-time PCR**—Quantitative real-time PCR was performed as described previously (23) using specific forward (5’-ATCGTTTCAAGGCGCACAG) and reverse (5’-H11032 CAGGTATCG) primers for cyclin G2. The fold change in expression was calculated using the ΔΔCt (threshold cycle) method as described (24), with the ribosomal protein 36B4 as an internal control (forward primer: 5’-GTGTTGAACAATGGCAGCAT; reverse primer: 5’-GACACCTCCTCAGGAAGCGA).

**Cyclin G2 Promoter Cloning**—Three fragments of the human cyclin G2 promoter (F1, −485 bp; F2, −1043 bp; and F3, −1605 bp) were obtained by PCR from MCF-7 cell genomic DNA using the Pfx kit (Invitrogen) and directionally cloned into the BglII and MluI sites of the pGL3basic reporter vector (Promega Corp.) according to standard subcloning techniques. The plasmids were sequenced to confirm the fragment insertion and sequence.

**Transient Cell Transfections and Site-directed Mutagenesis**—HeLa cells were transfected following Invitrogen’s protocol available online. The luciferase assay system used was purchased from Promega Corp. (Madison, WI). Luciferase values were normalized using a β-galactosidase gene-containing plasmid (pCMV/β, Clontech, Palo Alto, CA) as an internal control for transfection efficiency, as described previously (25).

All the plasmids used in the study were previously characterized by us or other laboratories (26–31). The amounts of plasmids used were: 1 μg of the reporter constructs, 100 ng of β-galactosidase, and 100 ng of estrogen receptor constructs. Every experiment was performed at least three independent times.

Site-directed mutagenesis was performed on the F1 fragment of the cyclin G2 promoter using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer’s instructions. The mutagenesis sense and antisense primers were designed using the Stratagene web site. The sequence for the sense primer was: 5’-GGCCGCGCG-GACCCGCGGAGCGTTTCAAGGCGCACAG (5’-Bio/CTAGATTACTTCTCTAGTTGCTGCGCAATGGCAGCAT; reverse primer: 5’-GTGTTGCAAAATGGCAGCAT; forward primer: 5’-GACACCTCCTCAGGAAGCGA).

**DNA Pulldown Assays**—Pulldown assays were carried out as described (32) using biotinylated oligonucleotides containing the vitellogenin A2 ERE (33) or a nonspecific sequence (34) or the cyclin G2 promoter sequence (5’-Bio/CTAGATTACTTCTCTAGTTGCTGCGCAATGGCAGCAT/GCGTTTCAAGGCGCACAG), a cyclin G2 promoter sequence with mutated half-ERE or a sequence containing only a half-ERE but no Sp1 binding sites (5’-Bio/CTAGATTACTTCTCTAGTTGCTGCGCAATGGCAGCAT/GCGTTTCAAGGCGCACAG). The plasmids were sequenced to confirm the mutation of the desired site.

**RESULTS**

**Cyclin G2 Is a Primary Target Gene Down-regulated by Estrogen**—Cyclin G2 was found to be an estrogen down-regulated gene in MCF-7 breast cancer cells in a microarray gene expression profiling study published by our laboratory (7). To investigate further the mechanistic basis for the estrogen-dependent regulation of cyclin G2, we first carried out a complete time course analysis with time points ranging from 30 min to 48 h. As shown in Fig. 1A, cyclin G2 mRNA was rapidly reduced to ~10% of the initial level by 2 h of treatment with 10 nM E2. Moreover, the levels of cyclin G2 mRNA remained at less than 40% of the initial level over the entire time course studied (Fig. 1A). A full dose-response curve at the 2-h time point was performed with E2 concentrations ranging from 10−12 to 10−6 M. Estrogen down-regulation was maximal at 10−10 M and above and showed an EC50 of ~3 × 10−11 M (Fig. 1B).

To confirm that the E2 hormone-evoked down-regulation was mediated through the ER, cells were treated with several ER agonist and antagonist ligands (Fig. 1C). The selective ERα agonist propyl pyrazole triol (35) also markedly down-regulated cyclin G2 expression, whereas treatments with the ER antagonists IC182,780 or trans-hydroxytamoxifen had no effect alone, and they prevented the E2-mediated down-regulation...
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FIGURE 1. 17β-Estradiol down-regulates cyclin G2 gene expression in MCF-7 human breast cancer cells. A, time course analysis after cell treatment with 10 nM 17β-estradiol. B, 17β-estradiol dose response monitored at 2 h of estradiol treatment. C, treatment with E2 (10 nM) or the ERα-selective ligand propyl pyrazole triol (10 nM) alone or with trans-hydroxytamoxifen (1 μM) or with ICI182,780 (1 μM) antiestrogen in the absence or presence of 10 nM E2. Ligand treatments in C were for 2 h. Values are the mean ± S.E. of at least three independent experiments. * p < 0.05 versus E2; **, p < 0.05 versus vehicle control.

FIGURE 2. Effect of cycloheximide (A) and trichostatin A and pathway inhibitors (B) on cyclin G2 mRNA. MCF-7 cells were treated with cycloheximide (CHX, 10 μg/ml) or with trichostatin A (TSA, 100 nM), LY294002 (LY, 10 μM), or gliotoxin (1.5 μM) alone (open bars) or in combination with 10 nM E2 (hatched bars) for 2 h. Values are the mean ± S.E. of at least three independent experiments. *, p < 0.05 versus no inhibitor vehicle + E2; **, p < 0.05 versus vehicle control.

FIGURE 3. Cyclin G2 promoter activity is down-regulated by ERα and mutation of the half-ERE in the cyclin G2 promoter F1 fragment abrogates the E2 down-regulation. A, schematics of human cyclin G2 promoter (~1.6 kb) with some of the putative transcription factor binding sites indicated. B, truncations (F1–F3) of the human cyclin G2 promoter or empty vector (pGL3basic) were transfected into HeLa cells along with ERα and β-galactosidase used as an internal control. C, the half-ERE site in the F1 promoter fragment was mutated using site-directed mutagenesis. The F1 wt and F1mut promoter constructs were transfected into HeLa cells, and promoter activity was assessed in the absence (control vehicle) or presence of 10 nM E2. Luciferase assay was performed after 24 h of E2 (10 nM) or vehicle treatment. Values are the mean ± S.E. of at least three independent experiments. *, p < 0.05 versus vehicle control.

Because cyclin G2 mRNA is down-regulated by E2 at early times, we evaluated whether any of these regions was necessary for E2-mediated down-regulation, indicating that the E2-ER complex has a direct effect on this gene and does not depend on other short-lived regulatory proteins (Fig. 2A).

To assess whether cyclin G2 is a primary ER gene target, we treated MCF-7 cells with the translational inhibitor cycloheximide (10 μg/ml). Cycloheximide treatment was not able to block E2-mediated cyclin G2 mRNA down-regulation, indicating that the E2-ER complex has a direct effect on this gene and does not depend on other short-lived regulatory proteins (Fig. 2A).

To evaluate whether any of these regions was necessary for E2-mediated down-regulation, these three fragments were isolated using a PCR-based approach and were directionally cloned in the luciferase reporter vector pGL3basic, which lacks promoter or enhancer elements. The three fragments, named F1, F2, and F3 and corresponding approximately to the −500, −1000, and −1600 bp fragments of the human cyclin G2 promoter, were transiently transfected along with ER into the ER-negative HeLa cell line. As shown in Fig. 3B, a 24-h treatment with E2 was able to significantly and reproducibly reduce by ~40–60% the activity of all the fragments tested, indicating that the 489-bp F1 fragment appeared to be responsible for the E2-mediated down-regulation. It is also evident that the longer fragments contain elements that are important for maintaining basal promoter activity (Fig. 3B, open bars), as a progressive reduction in activity was observed across the promoter deletions from F3 to F1.

Site-directed Mutagenesis Reveals a Role for the Half-ERE in the F1 Fragment of the Cyclin G2 Promoter—To evaluate the role of the half-ERE site present in the F1 promoter fragment (~−500 bp), we used site-directed mutagenesis to alter this site. We changed 4 out of 5 bases of the half-ERE to ensure that the altered binding site would not be recognizable by the ER and also to introduce a unique SacII restriction site to facilitate colony screening. Transient transfections were performed in HeLa cells with multiple independent clones containing the desired mutation. We found that all of the half-ERE mutated clones (F1mut) were completely unresponsive to E2 (Fig. 3C and data not shown). This result indicates that the half-ERE element in the cyclin G2 F1 promoter region is necessary for repression by the E2-ER.
different ERα constructs containing deletions or mutations known to affect distinct functions of the receptor. Two dominant negative ERα mutants (Fig. 4), containing a point mutation (L540Q) or a frameshift mutation (S554fs) that abrogate AF-2 activity and coactivator binding but maintain ligand binding (30, 31, 36), both showed a greater down-regulation of cyclin G2 promoter activity in response to E2 (to ~20–30% of control activity) than observed for the wild type ERα (~50% of control basal activity, Fig. 4), indicating that a functional wild type AF-2, which is fundamental for coactivator binding, is not necessary for this down-regulation and might in fact be masking a repressor surface present in the C-terminal portion of the receptor (37, 38) that becomes more exposed in these dominant negative ERs.

A triple point mutation in the DNA binding domain (DBDtm) that greatly reduces the affinity of the receptor for ERs completely abrogated the hormone elicited down-regulation, indicating that a functional DNA binding region is necessary for the ER-mediated repression. Likewise, deletion of the A/B domain (CDEF, which lacks the whole A/B domain) or deletion of only the first 21 amino acids of the receptor (N21) abrogated the repressive effect of estrogen, indicating an essential role for the N-terminal A domain (Fig. 4), a region shown to be important in interaction with the C-terminal part of the receptor (39, 40). Since all of the receptors examined were expressed at similar levels, as seen in Fig. 4 and published for some of these receptors previously (30, 31, 36), the differences in receptor-repressive activity are not attributable to alterations in cellular receptor levels.

Hence, regional mapping of ERα indicates an important role for the N-terminal region and the DNA binding domain in the repression of the cyclin G2 promoter and an enhanced repressive activity of dominant negative ERs with mutations in activation function-2 known to result in loss of coactivator binding (30, 41).

**ERα Is Recruited at the Cyclin G2 Promoter in Vivo, and RNA Polymerase II Is Dismissed from the Cyclin G2 Promoter upon Estrogen Treatment**—To examine ER recruitment to the cyclin G2 promoter in MCF-7 cells in vivo, we used ChIP assays. The cells were treated for 45 min with either control vehicle or 10 nM E2, after which chromatin was cross-linked with formaldehyde, and protein-DNA complexes were immunoprecipitated with antibodies to ERα, RNA polymerase II, or anti-acetyl (K16)-histone H4. The PCR primers used encompass the half-ERE site in the cyclin G2 promoter (5 kb). At this ERE site, the p52 promoter, which is stimulated by the E2-ER complex and contains an imperfect ERE (42), was used as a positive control, and an unrelated region ~5 kb upstream of the translational start site of the cyclin G2 promoter was used as a negative control.

As shown in Fig. 5A, ERα was recruited to the cyclin G2 promoter in a ligand-dependent manner. ERα was also recruited to the p52 promoter but not to the unrelated (cyclin G2, ~5 kb) region, as expected. RNA polymerase II was released from the cyclin G2 promoter upon E2 treatment, and histone H4 became deacetylated, indicating that with the E2-ER interaction, the chromatin in this region becomes a less permissive environment for transcription. A very different situation was seen at the p52 promoter, where the ER acts as a transcriptional activator. Here, E2 treatment of cells resulted in recruitment of RNA polymerase II, accompanied by an increase in acetylated histone H4 (Fig. 5A).

*An N-CoR-HDAC-1-containing Complex Is Recruited at the Cyclin G2 Promoter*—Because we found that the repressive effect of estrogen on cyclin G2 gene expression was reversed by the histone deacetylase inhibitor trichostatin A and that ERα recruitment led to a hypoacetylated chromatin at the cyclin G2 promoter, as candidate mediators of ER action we first looked for histone deacetylases, in particular the class I HDAC-1 and HDAC-3, and the class II HDAC-4, -5, and -7 enzymes, as it is known that different corepressor complexes contain one or more HDAC proteins (43). In Fig. 5B, ChIP analyses on MCF-7 cells show that, after E2 treatment, HDAC-1 is robustly enriched and the class II HDACs are slightly enriched at the cyclin G2 promoter. HDAC-3 was present at the promoter, and its level was not affected by ligand treatment. In contrast, none of the HDACs was found either at the p52 promoter or at the ~5 kb (control) region of cyclin G2 before or after E2 treatment.

To examine possible corepressor complex recruitment by the E2-ER at the cyclin G2 promoter, we tested the central subunits of the N-CoR/SMRT complexes, N-CoR and SMRT (43–45), and SAP Sin3A (46, 47) by ChIP assays (Fig. 5C). Of note, N-CoR showed hormone-dependent recruitment to the cyclin G2 promoter (but not to the p52 promoter or ~5-kb cyclin G2 region), highlighting a possible new role for this corepressor in down-regulation of gene expression by the E2-ER complex. SMRT was weakly detected after E2 treatment, and Sin3A was present, but its recruitment was not enhanced after hormone treatment.

**ERα and Sp1 Are Pulled Down from MCF-7 Nuclear Extracts Using Biotinylated Cyclin G2 Promoter Oligonucleotides**—Because the region responsible for ER-mediated repression of the cyclin G2 promoter is


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**FIGURE 6.** Examination of the interaction of Sp1 and ER with the cyclin G2 promoter and the importance of the half-ERE site. A, ChiP was performed in MCF-7 cells on the ER-responsive region of the cyclin G2 promoter using an Sp1 antibody or control IgG. The cells were treated for 45 min with either control vehicle or 10 nM E2. Similar results were obtained in multiple independent experiments. B and C, pulldown assays were carried out by incubating immobilized biotinylated oligonucleotides containing a nonspecific (NS) sequence, cyclin G2 sequence, the cyclin G2 sequence with a mutated half-ERE site (Mut 1/2ERE), a sequence containing only a half-ERE (TGACC, 1/2ERE), or the vitellogenin A2 ERE (VitA2) with nuclear extracts from E2 treated MCF-7 cells. Specifically bound proteins were eluted and subjected to Western blot analysis using antibodies specific to Sp1 (B) or ERα (C). Left lane contains 20% of the input MCF-7 nuclear extract (NE), In C, only one half as much of the VitA2 eluate was loaded. The panels show data from one of three independent experiments with similar findings.

composed of a half-ERE embedded into a GC-rich region, we compared the recruitment of endogenous Sp1 and ERα at this site. By ChiP assays, Sp1 was found to be present at the cyclin G2 promoter regardless of hormone treatment (Fig. 6A). The presence of Sp1 and ERα at the cyclin G2-responsive region was further confirmed by DNA pulldown assays (Fig. 6, B and C). Nuclear extracts prepared from MCF-7 cells that had been treated with E2 were incubated with immobilized oligonucleotides containing a nonspecific (NS) sequence or the vitellogenin A2 ERE or 40 bp of the cyclin G2 F1 promoter fragment containing the half-ERE and GC-rich region. Specifically bound proteins were eluted and subjected to Western blot analysis using Sp1 or ERα antibodies. Sp1 interacted with cyclin G2 oligonucleotides encompassing the GC-rich and half-ERE sites but did not interact with either the vitellogenin A2 or nonspecific oligonucleotides (Fig. 6B). As shown in Fig. 6C, ERα could be detected in the positive control (vitellogenin A2 ERE) as well as in the cyclin G2 F1 promoter oligonucleotide sample but not in the negative control (nonspecific oligonucleotides), indicating that this 40-bp sequence of the cyclin G2 promoter is able to interact with the endogenous ERα of MCF-7 cells. An oligonucleotide containing a half-ERE site without any Sp1 binding sites recruited 75 ± 8% (n = 3 experiments) the amount of ER recruited to the natural cyclin G2 sequence (Fig. 6C). Moreover, interaction with ERα was reduced (to 52 ± 4.4% of wild type cyclin G2 sequence, n = 3) when the half-ERE was mutated (mut 1/2ERE), indicating the importance of the half-ERE site. These findings suggest the importance of both the half-ERE and the Sp1 sites in the recruitment of ER to the cyclin G2 promoter. They also reveal that Sp1 protein is likely involved in regulation of the basal expression of the cyclin G2 gene, since Sp1 is present in a ligand-independent manner (Fig. 6A) and might serve as a docking site for ER upon cell exposure to estradiol. A proposed model for estrogen-occupied ER repression of cyclin G2, and the involvement of Sp1 and a corepressor-HDAC complex, is presented under "Discussion."

**DISCUSSION**

**Cyclin G2, a Gene Robustly Down-regulated by Estradiol through the Estrogen Receptor**—The action of estradiol in its various target cells is regulated via estrogen receptor modulation of gene expression, either positively or negatively. In this report, we have examined the molecular events associated with hormone-induced negative regulation, which is much less well understood than positive regulation, by focusing on estrogen repression of cyclin G2 in breast cancer cells. Although repression of gene transcription by many transcription factors is known to be regulated by the recruitment of various multiprotein corepressor complexes, little is known about the nature of the molecular events by which steroid hormone-occupied nuclear receptors are able to suppress gene transcription.

Intriguingly, our findings with cyclin G2 indicate the recruitment of a corepressor complex by the promoter-bound estrogen-ER complex that induces hypoacetylation of histones, such as histone H4, thereby creating a repressive chromatin conformation associated with release of RNA polymerase II. Thus, on this gene the E2-ER functions as an inverse agonist, causing active repression of basal gene activity. In contrast, treatment of these cells with estrogen has the converse, agonist effect on activated genes such as pS2 (Fig. 6), where RNA polymerase II is recruited and histone acetylation is increased by E2-ER.

It is of note that N-CoR was preferentially recruited to the cyclin G2 promoter by the E2-occupied ER, but SMRT showed little recruitment. Moreover, HDAC-1 was the most strongly recruited HDAC following estrogen treatment of cells. Other HDACs might also be involved, since we detected HDAC-3 and class II HDACs (recognized by a pan-specific antibody). However, HDAC-3 was clearly found at the promoter in a ligand-independent manner, and class II HDACs were only slightly enriched upon E2 treatment. Likewise, Sin3A was present in a ligand-independent manner. Thus, mechanistically, the molecular events in the active repression of cyclin G2 by E2-ER parallel those that follow antiestrogen suppression of genes that are normally estrogen-activated, such as pS2 and c-myc (42, 48, 49).

Although N-CoR and SMRT were first cloned on the basis of their interactions with unliganded retinoic acid and thyroid hormone receptors, they are now documented to serve as corepressors for a variety of unrelated transcription factors such as AP-1, Pit-1, and Oct-1 (43) and for antagonist-occupied estrogen and progesterone receptors. It is of note that although we observed the recruitment of the corepressor N-CoR to the cyclin G2 gene, we observed little or no recruitment of the related corepressor, SMRT. Our results are consonant with evidence that these two corepressors have different preferences and determinants for interactions with nuclear receptors and different transcription factors at specific genes (43, 50) and hence might have different selectivities for interacting with the estrogen-ER complex at this gene. A prior study on the estrogen-down-regulated E-cadherin gene showed N-CoR at the gene promoter, but no change in N-CoR abundance was reported with estrogen treatment (51). Very recently, during the review of our manuscript, Zhu et al. (52) reported the recruitment of N-CoR to the promoter of three genes repressed by estradiol, supporting the observations we have made for cyclin G2. Neither (51, 52) studied the involvement of HDACs, histone acetylation, or other cofactors such as Sp1 in the gene regulations.

A Model for Gene Repression Mediated by ER-Agonist Complexes; Comparison with Repression by Antiestrogens—Through promoter analyses and deletion and mutational studies, we have shown that the E2-responsive region of cyclin G2 is in the first 500 bp upstream of the ATG, which contains a half-ERE site clustered in a GC-rich region. Mutation of the half-ERE greatly reduced ER binding and abrogated E2-mediated repression. GC-rich regions are known to be involved in ER-mediated repression at the p21/WAF1 and VEGF gene promoters, where interplay with members of the Sp1 family of transcription factors seems to occur (53). Furthermore, direct ER and Sp1 binding is well documented in estrogen-stimulated genes (54). Indeed, through DNA pulldown and chromatin immunoprecipitation experiments, we were able to show that both Sp1 and ERα bind to the responsive region of the cyclin G2 promoter. Of note, E2 treatment markedly enhanced ER association, whereas Sp1 was present both in the presence or absence of E2 at the
cyclin G2-responsive region, resembling the situation described at the p21/WAF1 promoter (53). In their study, Varshoshi et al. (53) hypothesized that Sp1 might be responsible for ERα and HDAC-1 recruitment at the p21/WAF1 promoter to maintain its repressed state.

Our proposed model for ERα-mediated repression of the cyclin G2 gene is depicted in Fig. 7. In the absence of estradiol, cyclin G2 basal regulation seems to be mediated via multiple regulatory regions, as shown from the promoter deletion analysis, in which factors such as Sp1 and possibly others are involved (55). These factors could control cyclin G2 expression by acting as transcriptional activators, recruiting an activator complex that stabilizes the basal transcriptional machinery at the cyclin G2 promoter, thus enabling a permissive chromatin conformation and gene transcription. Upon E2 treatment, ERα is recruited by Sp1 to the half-ERE-containing region, and here it causes displacement of RNA polymerase II and induces recruitment of a corepressor complex containing N-CoR and histone deacetylases. Formation of this complex leads to hypo-acetylation of histones, which causes stabilization of the nucleosome structure, limiting accessibility to the basal transcriptional machinery and thus repressing cyclin G2 gene expression.

It is of note that this recruitment of distinct chromatin-modifying complexes by the estrogen-bound ER and the dismissal of RNA polymerase II at the cyclin G2 gene promoter in vivo is highly reminiscent of the scenario of a similar recruitment of chromatin-modifying complexes by the tamoxifen-occupied ER at genes that are repressed by this tissue selective estrogen receptor modulator, acting as an antiestrogen in breast cancer. These studies (42, 48, 49) have revealed the recruitment of an N-CoR-HDAC complex at the pS2 and the c-e2 promoter site. BTM, basal transcriptional machinery; Ac, acetylated histones.

DNA regulatory region/nuclear hormone receptor composite surface is crucial for enabling the estrogen-liganded ER to recruit N-CoR protein and associated HDACs that result in transduction of repression signals involving histone deacetylation and alterations in chromatin structure and/or direct interactions with the basal transcriptional machinery to evoke the hormone-induced negative regulation observed at the cyclin G2 gene. Whereas repression of some target genes negatively regulated by the glucocorticoid receptor appears to be mediated by negative glucocorticoid response elements that are distinct from glucocorticoid response elements in stimulated genes (60), there is thus far no clear evidence for involvement of equivalent negative EREs in gene repression by the E2-occupied ER.

Our findings highlight the importance of not only the hormone binding domain but also the N-terminal A domain of the ER and the receptor DNA binding domain in the ability of the ER to suppress cyclin G2 gene expression. These findings support prior evidence for important interactions of the N- and C-terminal regions of this and other nuclear receptors in their regulation of gene expression (39, 40, 61) and in particular the N-terminal A region of the receptor in the repressive actions of antiestrogens (62) and dominant negative ERs (30). The region of the A domain required for cyclin G2 repression appears to be at the far N terminus because the first 21 amino acid portion of the A domain was crucial for cyclin G2 repression by the estrogen-occupied ER.

Diverse Patterns of Gene Regulation by Estrogens through the Estrogen Receptor—Because exposure of cells to estrogen has a major impact on gene expression, with the activity of many genes being affected (greater than 1% of human genes appear to be primary targets of estrogen action, about half of which are repressed) (7), it is likely that there will prove to be several mechanisms responsible for gene repression. While our study has highlighted the role of active repression via N-CoR and HDACs in the regulation of the cyclin G2 gene, other mechanisms of repression might also contribute, such as sequestration of limiting stimulatory factors (squelching) away from certain genes to those that are being activated by estrogen.

In the studies reported here, we establish that the estrogen-occupied ER can actively recruit a nuclear corepressor-HDAC complex to a gene at which this hormone exerts negative regulation. This scenario allows the receptor to carry out its program of negative regulation, which along with the ability of these estrogen-occupied ERs to work at other genes to enact hormone-induced positive regulation, permits an integrated pattern of gene regulation that underlies the ability of this sex-steroid hormone to control important gene and cellular activities in breast cancer.

Because our studies and those of others indicate that repression of gene expression by estrogen plays as central a role as does stimulation of gene expression in the actions of sex steroid hormones in normal as well as cancer target cells (7, 51, 63, 64), a greater understanding of the mechanisms and proteins involved, as are beginning to be elucidated by this study, should provide new insights and reveal potential therapeutic strategies. This study is one of the first steps toward understanding the way in which the estrogen-ER complex can attenuate gene activity. Future studies will be required to fully characterize the multiple repression mechanisms that likely underlie ER function at its many repressed target genes.

Acknowledgment—We thank Jeanne Danes for excellent technical assistance.

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