

# Dominant-Negative Nuclear Receptor Corepressor Relieves Transcriptional Inhibition of Retinoic Acid Receptor but Does Not Alter the Agonist/Antagonist Activities of the Tamoxifen-Bound Estrogen Receptor

ASHBY J. MORRISON, RAFAEL E. HERRERA, ESTER CORONADO HEINSOHN, RACHEL SCHIFF, AND C. KENT OSBORNE

*Department of Molecular and Cellular Biology and The Breast Center (A.J.M., R.E.H., C.K.O.) and Department of Medicine and The Breast Center (R.S., C.K.O.), Baylor College of Medicine, Houston, Texas 77030; and Department of Medicine (E.C.H.), University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284*

Repression of the transcriptional activities of the estrogen receptor (ER) is a main goal in the treatment of breast cancer. The antiestrogen tamoxifen is an effective therapy for breast cancer patients because it inhibits estrogen-stimulated gene expression and cell proliferation. Previous studies have implicated a complex containing the nuclear receptor corepressor (N-CoR) in the mechanism by which tamoxifen represses ER-mediated transcriptional activity. In the present study a truncated N-CoR construct was used to inhibit endogenous N-CoR activity in an ER-positive breast cancer cell line. This dominant-negative N-CoR was successful in relieving repression conferred by the unliganded retinoic acid receptor, but it failed to affect the transcriptional activity of the ER in the presence of

tamoxifen. Correspondingly, the histone acetylation levels of nucleosomes on endogenous estrogen-responsive genes were unaltered in cells expressing the N-CoR dominant-negative, regardless of ligand. In addition, *in vitro* cell proliferation and *in vivo* tumor growth were unchanged in cells that express dominant-negative N-CoR. In conclusion, these results may reveal that N-CoR affects tamoxifen-liganded ER in a manner distinct from its influence on retinoic acid receptor-mediated transcriptional activity or that corepressors other than N-CoR may be involved in the ability of tamoxifen to repress estrogen-responsive transcription and tumor growth. (*Molecular Endocrinology* 17: 1543–1554, 2003)

THE ESTROGEN RECEPTOR (ER) is a member of a large family of nuclear hormone receptors that activate transcription of specific genes when ligand binds to the receptor and the complex binds to specific DNA sequences of target genes (1). The ER plays an important role in a variety of physiological cellular functions, including estrogen-induced cell proliferation. As a result, activation of the ER significantly contributes to the development and progression of breast cancer, and ER is a useful diagnostic and therapeutic target (2, 3).

One of the most widely prescribed drugs for the treatment of breast cancer is the antiestrogen tamoxifen, a selective ER modulator that displays both estrogen agonist and antagonist activities that can be

species, tissue, or gene specific (4, 5). In breast tissue, tamoxifen binds to the ER and functions as an antiestrogen by inhibiting expression of genes required for cell proliferation (6). Although tamoxifen is an effective therapeutic agent for the treatment of breast cancer, most tumors eventually become resistant to treatment through mechanisms that are not completely clear. One potential resistance mechanism that is supported by compelling laboratory and clinical data is the acquired ability of tamoxifen to stimulate rather than to inhibit tumor growth, perhaps via enhancement of its intrinsic agonist activity (7).

Recent evidence has shown that the varying effects of estrogen and antiestrogen-liganded ER may be due to the activity of transcriptional coactivators and corepressors that bind to receptor (8, 9). Several ER-interacting coactivators have previously been found to bind ER when liganded to estrogen, such as SRC-1/N-CoA1 (10), GRIP1/TIF2/N-CoA2 (11, 12), CBP/p300 (13), and AIB1/p/CIP/RAC3/ACTR (14–17). In addition, several transcriptional coregulators have been reported to associate with and alter the activity of antagonist-bound steroid hormone receptors. The co-

Abbreviations: ChIP, Chromatin immunoprecipitation; ER, estrogen receptor; ERE, estrogen-responsive element; GST, glutathione-S-transferase; IVT, *in vitro* translated; MTS, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; N-CoR, nuclear receptor corepressor; RAR, retinoic acid receptor; RARE, retinoic response element; SMRT, silencing mediator for retinoid and thyroid hormone receptors; SRC, steroid receptor coactivator; TR, thyroid hormone receptor.

activator known as switch protein for agonists (L7/SPA) increases the agonist activity of the tamoxifen-bound ER (18).

Conversely, the antagonist activity of the tamoxifen-occupied ER may be enhanced by corepressor proteins. The corepressors referred to as nuclear receptor corepressor (N-CoR) and silencing mediator for retinoid and thyroid-hormone receptors (SMRT) were first identified as components of a complex that is involved in the active repression associated with unliganded retinoic acid receptor (RAR) and thyroid hormone receptor (TR) (19, 20). These proteins also have been reported to interact with antagonist-occupied steroid hormone receptors, resulting in an increase in the transcriptional repressive activities of the receptors (18, 21–24). Although a direct tamoxifen-dependent interaction of N-CoR with ER has not been consistently demonstrated, the corepressor N-CoR was shown to bind the promoter region of tamoxifen-occupied ER target genes (25, 26). Additionally, microinjection of N-CoR into N-CoR<sup>-/-</sup> mouse embryo fibroblasts resulted in a decrease in transcriptional activation of ER in the presence of tamoxifen (24). However, recent data that contrast the previous findings have determined that the receptor interaction domains of N-CoR and SMRT do not associate with estrogen receptor in a library screen or mammalian two-hybrid assay, whereas they do interact with TR and RAR (27). These results imply the potential participation of corepressors other than N-CoR and SMRT in tamoxifen-mediated ER repression, or the possibility that to bind ER these corepressors require regions other than the receptor interaction domains necessary for association with RAR and TR. Nevertheless, it has been demonstrated that inhibiting the activity of N-CoR or the corepressor complex by intracellular injections of blocking antibodies increases the agonist properties of tamoxifen (23, 28). Furthermore, in a xenograft model of tamoxifen resistance, which is caused by acquired tamoxifen-stimulated growth, we found that N-CoR protein levels were decreased coincident with the emergence of resistance and tumor regrowth, suggesting that reduced N-CoR might contribute to this response (23, 29).

These data led to the hypothesis that the N-CoR corepressor complex that associates with tamoxifen-bound ER may be important for the antagonist effects of the drug. Furthermore, loss of N-CoR activity might contribute to the onset of tamoxifen resistance by increasing its agonist effects, causing growth stimulation rather than suppression. In the present study, we have further examined the role of N-CoR in ER-dependent estrogen/antiestrogen action by expressing a truncated N-CoR, which functions as dominant-negative mutant N-CoR, to reduce N-CoR function in human breast cancer cells, the therapeutic target of tamoxifen. We found that N-CoR and the dominant-negative N-CoR are able to bind both ER and RAR *in vitro*. However, inhibiting endogenous N-CoR activity does not alter the ability of ER to repress target genes

in the presence of tamoxifen, whereas it is able to relieve repression of RAR in the absence of ligand. In addition, the *in vitro* and *in vivo* proliferation of the breast cancer cells expressing the N-CoR dominant-negative remain unchanged when stimulated by estrogen or inhibited by tamoxifen. These differing effects of the dominant negative N-CoR on ER and RAR activity may suggest that the mechanism of association between N-CoR and tamoxifen-bound ER may differ from its association with RAR. However, it may also allude to the possibility that corepressors other than N-CoR are involved in the ability of tamoxifen to repress estrogen-mediated transcription and proliferation.

## RESULTS

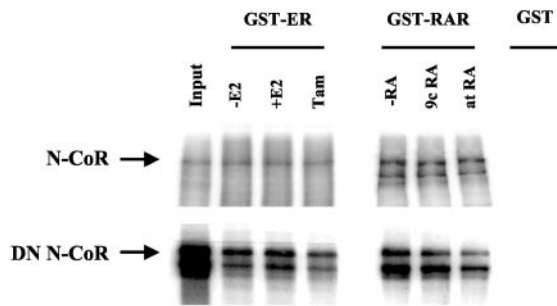
### Dominant-Negative N-CoR Interacts with ER *in Vitro*

As previously reported by Soderstrom *et al.* (30), the dominant-negative N-CoR mutant used in this study effectively interfered with endogenous N-CoR activity. This truncated N-CoR construct encodes amino acids 1586–2211 of the full-length protein. It blocks corepressor activity by retaining receptor interaction domains while lacking critical repressor domains as well as domains necessary for interaction with other components of the corepressor complex.

To determine the ability of the dominant-negative N-CoR to effectively alter the activity of tamoxifen-bound ER, we first performed interaction studies using *in vitro* translated (IVT) full-length and dominant-negative N-CoR protein and bacterially produced glutathione-S-transferase (GST)-ER fusion protein. It has been previously shown that the carboxy terminus of N-CoR containing amino acids 2057–2453 can bind to ER in a ligand-independent manner *in vitro* (22). The GST pull-down assay in Fig. 1 shows that the dominant-negative N-CoR used in this study can also associate with the ER in a manner similar to that of full-length N-CoR. As a control, the IVT N-CoR products were incubated with the RAR. As expected, both dominant-negative N-CoR and full-length N-CoR also associated with the GST-RAR fusion protein (Fig. 1). However, the biological relevance of this *in vitro* binding assay remains questionable considering the ligand-independent binding of both full-length and dominant-negative N-CoR with ER, as well as the ligand-independent binding of full-length N-CoR with RAR.

### Transient and Inducible Expression of Dominant-Negative N-CoR Does Not Alter the Ability of Tamoxifen to Inhibit ER-Mediated Transcription

We then used transient transfection assays to investigate the effect of full-length N-CoR and the dominant-negative mutant on the transcriptional activity of antagonist-occupied ER. Full-length and dominant-



**Fig. 1.** *In Vitro* Interaction of N-CoR and Dominant-Negative N-CoR in GST Pull-Down Assays

IVT full-length N-CoR and dominant-negative N-CoR (DN N-CoR) interact with bacterially produced GST-ER $\alpha$  and GST-RAR $\alpha$  fusion proteins. One tenth of IVT input is shown in lane marked "Input." N-CoR and dominant-negative N-CoR interaction assays with ER was performed in the presence of vehicle alone (–E2), with estradiol (+E2), or with tamoxifen (Tam). N-CoR and dominant-negative N-CoR association with RAR was performed in the presence of vehicle alone (–E2), 9-*cis*-retinoic acid (9cRA), or with all-*trans*-retinoic acid (atRA).

negative N-CoR constructs were transiently transfected into the estrogen-responsive MCF7D breast cancer cell line along with a reporter construct composed of the estrogen-responsive pS2 promoter that contains a consensus estrogen-responsive element (ERE) (31). The cotransfection assay shows that these constructs failed to significantly relieve repression on the pS2 promoter in cells treated with tamoxifen (Fig. 2A). In addition, these N-CoR constructs showed no effect on the ER in the absence of ligand, in the presence of estrogen, or in the presence of both estrogen and tamoxifen (Fig. 2A and data not shown). Furthermore, a dose response of full-length and dominant-negative N-CoR ranging from 30–500 ng of vector DNA was used in these transient transfection assays. Although a general decrease in transcriptional activation occurred when higher amounts of plasmid were transfected into the cell, this effect appears to be nonspecific and can be observed when vector alone is transfected (Fig. 2B). In conclusion, Fig. 2B shows that in this dose response assay no effect is seen on the transcriptional activity of the ER even when the highest concentration of N-CoR construct is cotransfected into the cell. However, the dominant-negative N-CoR was able to increase the transcriptional activity from a reporter construct containing a natural retinoic acid response element (RARE), indicating that it was expressed and functional (Fig. 2C). Thus, the dominant-negative N-CoR relieved repression on an unliganded nuclear hormone receptor but not on antagonist-bound ER. These data suggest that N-CoR does function to maintain the repression of unliganded RARs as expected, but it appears to be less important for the antagonist activity of tamoxifen-bound ER.

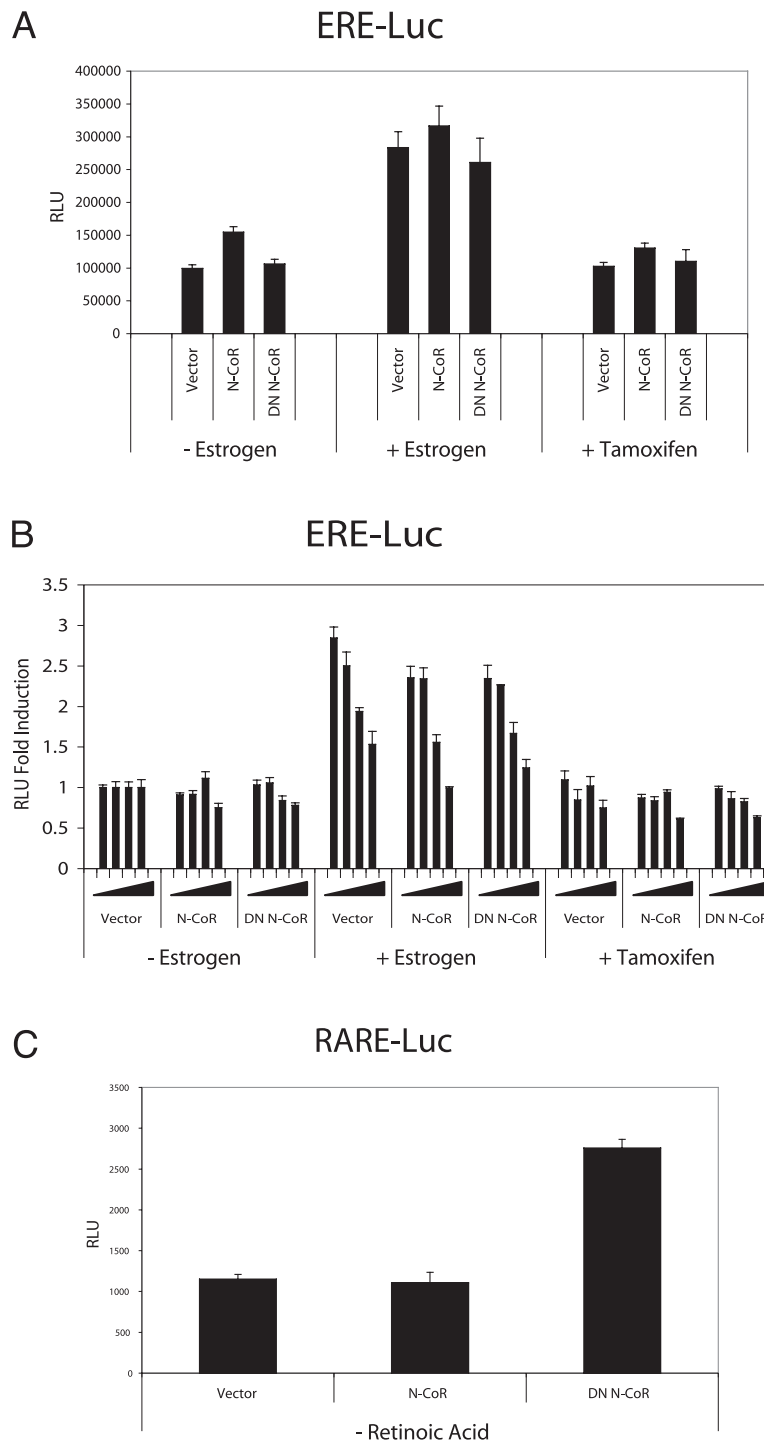
To further investigate the role of N-CoR on antagonist-liganded ER, we developed an inducible system

with the dominant-negative N-CoR construct. MCF7D cells were used to implement the inducible Dimerizer system (Ariad Pharmaceuticals, Cambridge, MA). This system is based on the constitutive expression of transcription factors that dimerize and induce transcription of a target gene upon exposure of the cells to a synthetic ligand. We created an inducible N-CoR dominant-negative construct that was stably integrated and displayed strong inducible expression upon addition of dimerizer in clonal cell lines (Fig. 3A). In Fig. 3B we show the effects of RAR transcriptional activity with and without inducer on a RARE reporter construct. When a RARE reporter gene was transfected into the cell, an approximate 7-fold increase in transcription occurred on the RAR-responsive gene in the absence of ligand but in the presence of the dominant-negative N-CoR when compared with MCF7D control cells. Transcriptional induction also occurred when the cells were in the presence of retinoic acid, although only a modest additional increase in transcriptional activity occurred when the dominant-negative N-CoR was simultaneously expressed in these cells (data not shown). The experiments presented in Fig. 3B provide additional evidence for the effectiveness of the dominant-negative N-CoR in relieving the transcriptional repression that is conferred by N-CoR. However, while addition of estrogen increased transcriptional activity on an ERE reporter construct and tamoxifen reduced this activation, induction of the dominant-negative N-CoR had little effect on the transcriptional activity occurring on the vitellogenin promoter by ER in the presence of tamoxifen (Fig. 3C). Although an approximate 2.5-fold increase in transcription occurs in one cell line (DN N-CoR-18) in the absence of ligand and when dominant-negative N-CoR expression is induced compared with non-induced cells (Fig. 3C), this effect is only observed in this cell line and may be due to higher levels of basal transcription in general.

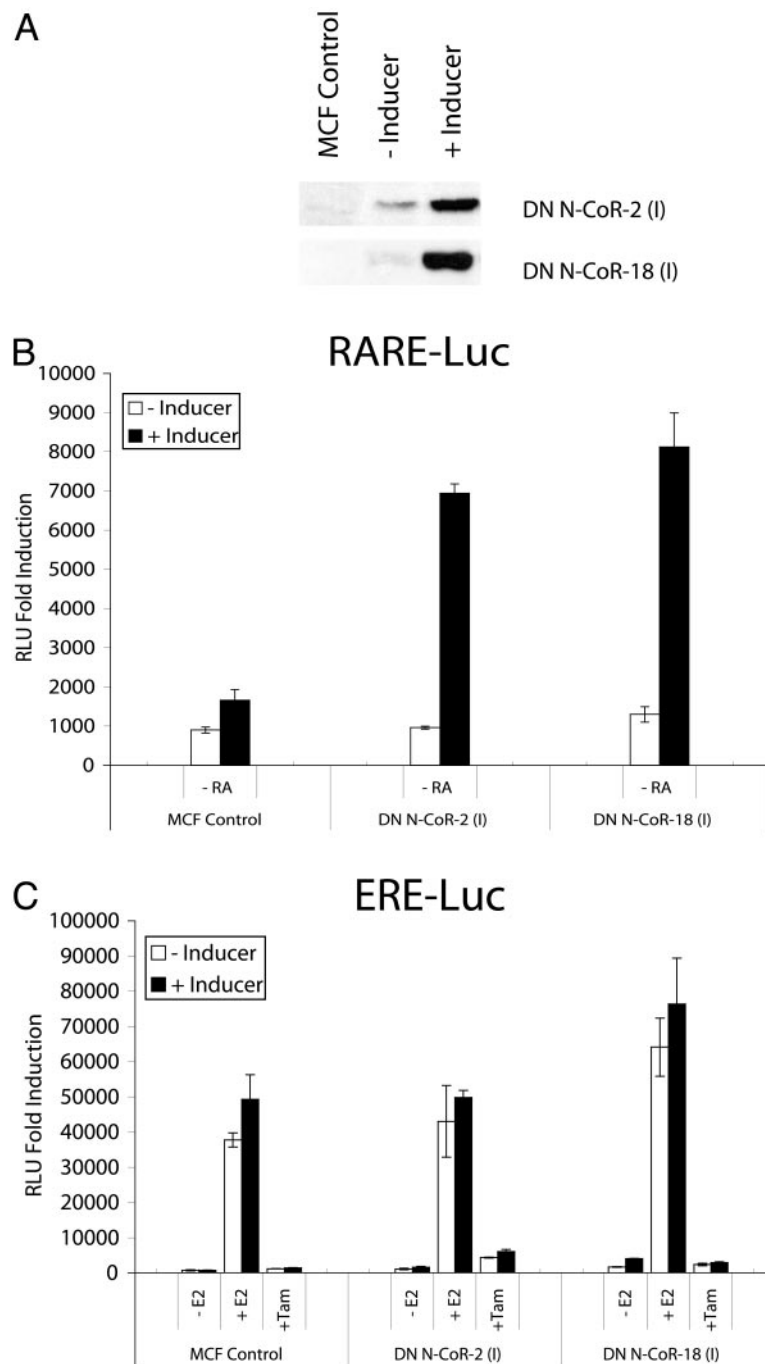
In summary, both the transient transfection as well as the inducible experiments show that dominant-negative N-CoR does not increase the agonist potential of tamoxifen on two different naturally occurring ERE-containing genes, the vitellogenin and pS2 genes.

#### **Dominant-Negative N-CoR Expression Fails to Alter the Transcriptional Repressive Effects of Tamoxifen on Endogenous Genes**

Although the previously described results do not support the hypothesis that the activity of an N-CoR corepressor complex is necessary for the antagonist properties of tamoxifen, these observations are not entirely conclusive. Reporter constructs that are transiently transfected into cells may not adopt an organized nucleosomal structure and consequently may not be an accurate system by which to analyze the function of chromatin-altering proteins such as transcriptional corepressors. Therefore, we developed cells that con-



**Fig. 2.** Effect of N-CoR and Dominant-Negative N-CoR Transient Overexpression on the Transcriptional Activity of ER and RAR In MCF7D cells: A, pS2-luciferase (ERE-Luc) cotransfected with 250 ng full-length N-CoR, dominant-negative mutant N-CoR (DN N-CoR), or vector alone in the presence of vehicle alone, estradiol ( $10^{-9}$  M) or the antiestrogen 4-hydroxytamoxifen ( $10^{-7}$  M); B, 30, 75, 150, or 500 ng of full-length N-CoR, dominant-negative mutant N-CoR (DN N-CoR), or vector alone in the presence of vehicle alone, estradiol ( $10^{-9}$  M) or the antiestrogen 4-hydroxytamoxifen ( $10^{-7}$  M). Results are displayed as fold induction by setting the –estrogen-treated cells transfected with vector within each dose group to 1; C, RAR $\beta$ -luciferase (RARE-Luc) cotransfected with 250 ng full-length N-CoR, dominant-negative mutant N-CoR (DN N-CoR), or vector alone in the absence of ligand. RLU, Relative luciferase units.



**Fig. 3.** Effect of Inducible N-CoR Dominant-Negative Expression on the Transcriptional Activity of ER and RAR

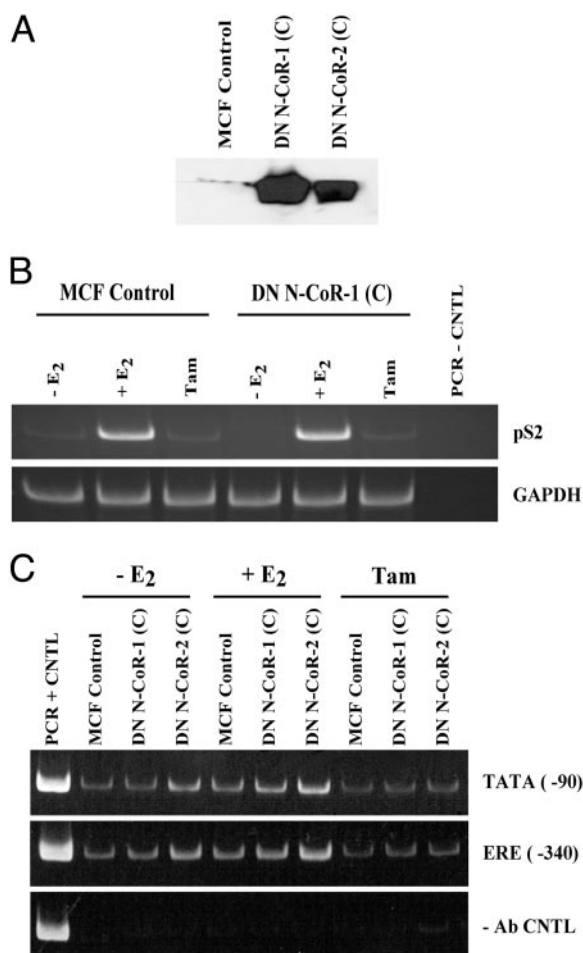
A, Western analysis of clonal cell lines expressing the inducible Flag-tagged dominant-negative N-CoR construct in two MCF7D clones, designated as DN N-CoR-2 (l) and DN N-CoR-18 (l). B, The RAR $\beta$ -luciferase (RARE-Luc) reporter construct was transfected into control cells and both MCF7D clones in the absence of ligand with inducible expression of the N-CoR dominant-negative (DN N-CoR). C, The vitellogenin-luciferase (ERE-Luc) reporter construct was transfected into control cells and both MCF7D clones with inducible expression of the N-CoR dominant-negative (DN N-CoR). Cells were treated with vehicle alone, estradiol ( $10^{-9}$  M), or 4-hydroxytamoxifen ( $10^{-7}$  M). RLU, Relative luciferase units.

stitutively express the dominant-negative N-CoR protein from a construct that was stably integrated into a breast cancer cell line. In these cells endogenous estrogen-responsive genes were examined for the modification of transcriptional response to tamoxifen, as

well as alteration of chromatin structure, as a result of loss of N-CoR activity.

The construct containing the dominant-negative N-CoR was introduced into the MCF7D breast cancer cell line. Stable clones were selected, many of which

expressed high levels of the dominant-negative N-CoR protein (Fig. 4A). Clones overexpressing full-length N-CoR could not be derived, either because of growth-inhibitory effects, which may be ER independent, and/or toxicity to the cell. Similar to the transient and inducible assay results, the dominant-negative N-CoR did not block the transcriptional inhibition mediated by tamoxifen on the endogenous pS2 gene as



**Fig. 4.** Analysis of Endogenous pS2 Transcription and Histone Acetylation in Cells Constitutively Expressing Dominant-Negative N-CoR

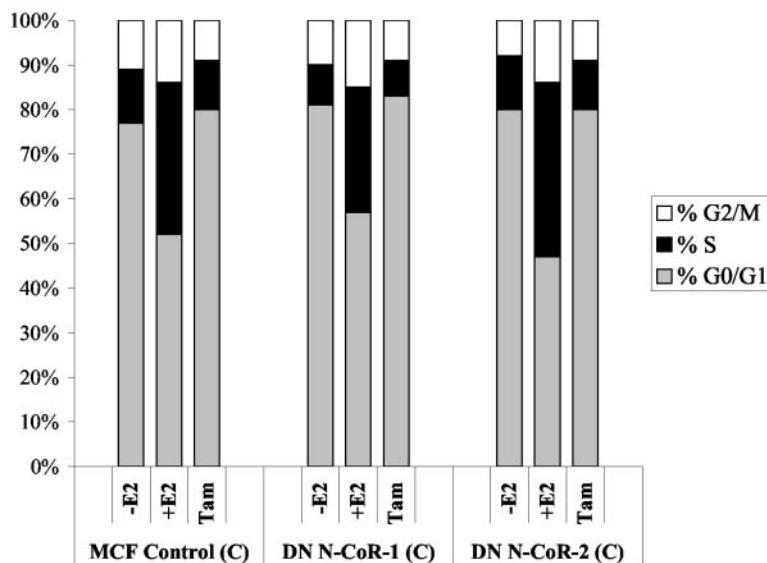
A, Western analysis of clonal cell lines constitutively expressing the Flag-tagged dominant-negative N-CoR construct in MCF7D cells, designated as DN N-CoR-1 (C) and DN N-CoR-2 (C). B, RT-PCR of mRNA in control cells and cells expressing dominant-negative N-CoR (DN N-CoR) treated with vehicle alone, estradiol ( $10^{-9}$  M), 4-hydroxytamoxifen ( $10^{-7}$  M), or 4-hydroxytamoxifen ( $10^{-7}$  M) plus histone deacetylase inhibitor, trichostatin A (TSA) (100 nM). Primers used were from either pS2 or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA sequences. C, ChIP analysis of histone acetylation of nucleosomes in the pS2 promoter. Control cells and clones expressing dominant-negative N-CoR (DN N-CoR) were treated with vehicle alone, estradiol ( $10^{-9}$  M), or 4-hydroxytamoxifen ( $10^{-7}$  M). PCR-amplified regions of the immunoprecipitated chromatin include the DNA region containing the ERE and the TATA box.

examined by RT-PCR (Fig. 4B). In this assay the general pattern of pS2 transcriptional activity was similar in the cells expressing the N-CoR dominant-negative and in the control MCF7D cells when treated with estrogen, tamoxifen, or vehicle alone.

Transcriptional coactivator complexes can greatly enhance the activity of the receptor through their intrinsic histone acetyltransferase activity (reviewed in Ref. 32). This acetyltransferase activity is presumed to alter the conformation of the chromatin on the target gene to provide a more accessible DNA template for the process of transcription (33, 34). Conversely, corepressor complexes have associated histone deacetylase activity that is involved in the inhibition of transcription (reviewed in Ref. 35). N-CoR is a part of a larger corepressor complex that contains histone deacetylase activity (36–41). We identified the pS2 gene as a good candidate to examine the chromatin alterations that may occur as a result of corepressor activity because the nucleosomes have previously been mapped on the pS2 promoter (42). Chromatin immunoprecipitation (ChIP) experiments utilizing MCF7D cells that constitutively express the dominant-negative N-CoR were performed to provide a functional analysis of the corepressor N-CoR by examining the acetylation status of the core histones in the pS2 promoter (Fig. 4C). If N-CoR and a protein conferring histone deacetylase are associated with tamoxifen-liganded ER, then interfering with this activity by expression of the dominant-negative N-CoR should result in an increase in histone acetylation. However, in the presence of the antagonist tamoxifen, expression of the dominant-negative N-CoR does not increase histone acetylation in the nucleosome positioned at the ERE or in the nucleosome positioned at the TATA box (Fig. 4C). Although there are some acetylation differences among the cell lines within each treatment group, because the cell lines are clonally derived direct comparisons are not made. Although it may be possible that the differences between the cell lines are informative, ChIP analyses alone cannot determine whether this is the case. In general, the pattern of histone acetylation correlates well with the transcriptional activity of the pS2 gene in the control cells and the dominant-negative N-CoR-expressing cell lines while in the presence of the agonist estrogen or in the presence of the antagonist tamoxifen.

#### Expression of Dominant-Negative N-CoR Does Not Alter the Inhibitory Effects of Tamoxifen on Breast Cancer Cell Proliferation

In the previously described assays, inhibition of N-CoR corepressor activity does not relieve transcriptional repression of the tamoxifen-bound ER on the genes examined. However, because the critical genes responsible for estrogen-mediated cell proliferation have not yet been identified, it is still possible that N-CoR may contribute to the antagonist effects of tamoxifen on estrogen-mediated cell proliferation. To



**Fig. 5.** Cell Cycle Analysis of Cells Expressing Dominant-Negative N-CoR

In MCF7D control cells and clones constitutively expressing the N-CoR dominant-negative, designated as DN N-CoR-1 (C) and DN N-CoR-2 (C), cell cycle analysis was determined by flow cytometry in cells treated with vehicle alone, estradiol ( $10^{-9}$  M), or 4-hydroxytamoxifen ( $10^{-7}$  M) for 48 h.

address this question, proliferation of MCF7D cells constitutively expressing a dominant-negative mutant N-CoR were analyzed for changes in cell cycle phase by DNA flow cytometry (Fig. 5). Similar to control cells, cells expressing the dominant-negative N-CoR are arrested in the  $G_0/G_1$  phase of the cell cycle in media lacking estrogen and are induced into S-phase with the addition of estrogen. In addition, both control and dominant-negative N-CoR-expressing cells become arrested in  $G_0/G_1$  after 48 h of treatment with tamoxifen. Similar results were obtained in proliferation experiments, in which cells were treated for 6 d with vehicle, estrogen, or tamoxifen. The proliferation rates of both control cells and cells that express dominant-negative N-CoR were stimulated by estrogen and inhibited by tamoxifen to the same extent (Table 1).

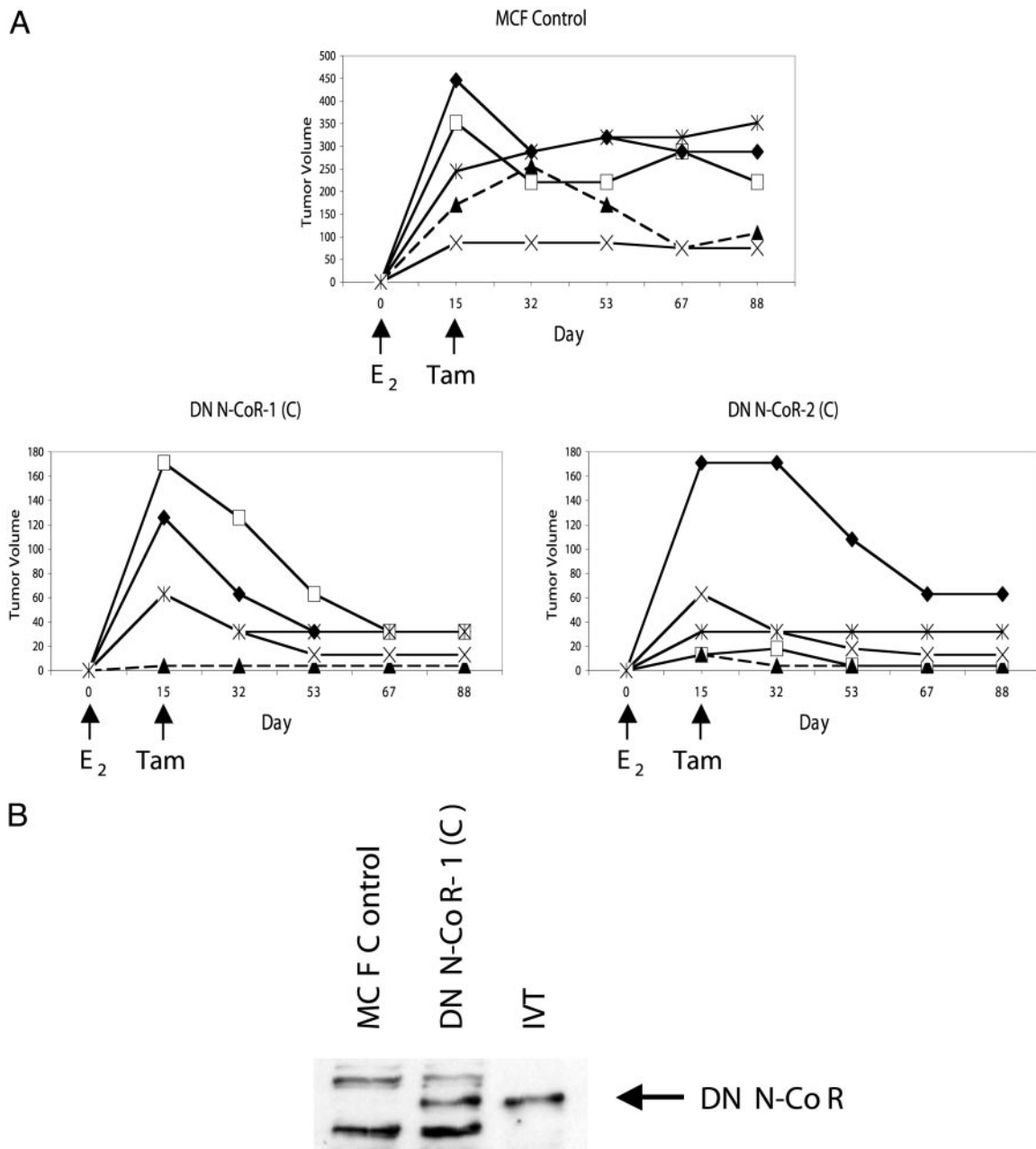
Stable dominant-negative N-CoR clones were also injected sc into the mammary fat pad of nude mice to monitor *in vivo* tumor growth. This system has been useful in examining the occurrence of tamoxifen-suppressed tumor growth (43). In these experiments, tumor development is initiated by estrogen treatment. After a short period, the estrogen pellet is removed and the mice are treated with tamoxifen, which causes an initial stabilization of the tumor followed by a decrease in tumor volume. This model predictably mimics the pattern of tamoxifen treatment in patients. We used this mouse model to examine the involvement of N-CoR on tumor growth. If N-CoR participates in the inhibitory activity of tamoxifen, then blocking its normal function with a dominant-negative mutant N-CoR would reduce the tumor growth-inhibitory effect. However, treatment of mice with tamoxifen inhibited growth of both control tumors and tumors expressing the dominant-negative N-CoR (Fig. 6A). Tumors in all

**Table 1.** *In Vitro* Proliferation Analysis of Cells Expressing Dominant-Negative N-CoR

Sample	Treatment	Absorbance (SD)
MCF control	-E <sub>2</sub>	0.503 (0.10)
	+E <sub>2</sub>	0.944 (0.08)
	Tam	0.560 (0.03)
dominant-negative N-CoR-1(C)	-E <sub>2</sub>	0.843 (0.12)
	+E <sub>2</sub>	1.651 (0.01)
	Tam	0.807 (0.14)
dominant-negative N-CoR-2(C)	-E <sub>2</sub>	0.743 (0.05)
	+E <sub>2</sub>	1.490 (0.16)
	Tam	0.795 (0.05)

In MCF7D control cells and clones constitutively expressing the N-CoR dominant-negative, designated as dominant-negative N-CoR-1(C) and dominant-negative N-CoR-2(C), MTS proliferation assay was performed in cells treated with vehicle alone, estradiol ( $10^{-9}$  M), or 4-hydroxytamoxifen ( $10^{-7}$  M) for 6 d. Relative proliferation rates are displayed as spectrophotometer absorbance units obtained from the MTS assay. SD of replicate samples shown in parentheses.

mice either stopped growing or regressed somewhat with the addition of tamoxifen. In the presence of estrogen (not shown), tumor volumes continue to increase as we have reported previously (43). Maintenance of expression of the N-CoR dominant-negative mutants was confirmed by Western analysis of tumor extracts obtained at the completion of the experiment (Fig. 6B). These *in vivo* experiments confirm the *in vitro* proliferation studies, suggesting that the dominant-negative N-CoR does not alter the effects of tamoxifen in these breast cancer cells.



**Fig. 6.** *In Vivo* Tumor Growth of Breast Cancer Cells Expressing Dominant-Negative N-CoR in Mouse Model of Tamoxifen Resistance

A, Tumor volume of control cells and clones expressing the dominant-negative N-CoR (DN N-CoR) in mice treated with tamoxifen after 15 d of estrogen-stimulated cell proliferation. Times of estrogen pellet insertion (E<sub>2</sub>) and tamoxifen administration (Tam) are noted on *graphs*. Each *line on the graph* denotes changes in size of a separate tumor. B, Flag Western analysis of harvested tumors consisting of MCF7D control cells and a clonal cell line constitutively expressing the DN N-CoR, designated as DN N-CoR-1 (C). IVT DN N-CoR is used as a control.

## DISCUSSION

It has been shown in a variety of cell lines from different tissues that the mixed agonist/antagonist activities of ER modulators, such as tamoxifen, can be modified by artificially changing the milieu of coactivators and corepressors. Expressing very high cellular levels of

the corepressors N-CoR or SMRT enhances the antagonist properties of tamoxifen-bound ER, whereas reducing corepressor activity or increasing levels of coactivators, such as SRC-1 and L7/SPA, increases agonist activity (18, 21, 24). Although these artificially high levels of coregulatory proteins may not be physiological, the data are consistent with the idea that

response to tamoxifen in patients with breast cancer may be influenced by the relative amounts of coactivator and corepressor complexes. Thus, an abundance of coactivator or a decrease in corepressor activity could contribute to tamoxifen resistance or even to tamoxifen-stimulated growth.

In a prior study, we demonstrated that the levels of N-CoR were markedly reduced coincident with the development of tamoxifen resistance (stimulated growth) in an *in vivo* human breast cancer model system (23). In addition, microinjection into cells of blocking antibodies against N-CoR or other members of the corepressor complex (Sin3, histone deacetylase, and Sin3-associated polypeptide, SAP30) converted tamoxifen into a full agonist on the ERE/LacZ reporter system (23, 28). Treatment with forskolin or epidermal growth factor also increased tamoxifen agonist activity, presumably by phosphorylation of ER, and thereby inhibited its interaction with N-CoR (23).

The data presented here are not supportive of the hypothesis that reduced N-CoR contributes to the development of tamoxifen-stimulated growth. To confirm the original results, we chose a different method of antagonizing N-CoR function that circumvents the need for microinjection of blocking antibodies into individual cells. We introduced a N-CoR dominant-negative deletion mutant that still binds ER but lacks important repression domains and domains required for interaction with Sin3. This mutant has been shown previously to relieve repression of unliganded thyroid hormone receptor (30). In this report we demonstrated *in vitro* interaction of both the full-length and a dominant-negative N-CoR with ER, but in a ligand-independent manner. We then used several methods of introducing this dominant-negative construct into MCF7 breast cancer cells, including transient transfection, inducible expression, and also stable constitutive expression, to show that while the levels of expression of this mutant were sufficient to relieve transcriptional repression on RAR, no significant effects were seen with ER, either bound to tamoxifen or estrogen or in the absence of ligand.

These conflicting effects of the dominant-negative N-CoR on ER and RAR transcriptional activity may suggest that the dominant-negative N-CoR interacts with ER differently than it interacts with RAR, or implicate the involvement of other corepressors in tamoxifen-mediated transcriptional repression. The data obtained from transcription analyses are similar for both the dominant-negative and full-length N-CoR, in that the transfected construct has no tamoxifen-specific transcriptional effect in MCF7 cells. In accord with the results observed in the transcription experiments, inhibiting N-CoR-associated deacetylase activity by expressing the N-CoR dominant-negative did not result in an increase in histone acetylation of nucleosomes in an estrogen-responsive gene. Finally, *in vitro* and *in vivo* tumor growth responses to estrogen and tamoxifen were unaltered by inhibiting N-CoR activity via dominant-negative N-CoR expression. These results

are consistent with our most recent observation that N-CoR protein levels in N-CoR extracts of primary breast cancer do not predict benefit in patients treated with tamoxifen adjuvant therapy, whereas high levels of the coactivator AIB1 (SRC3) strongly predict resistance to the drug (44, 45). They also support recent evidence showing that peptides encoding receptor-interacting domains that contain the CoRNR consensus motif of both N-CoR and SMRT do not interact with ER regardless of ligand, although they do interact with unliganded TR and RAR, as well as antagonist-bound progesterone receptor (27). These results and those presented in this report suggest the possible involvement of other corepressors in the transcriptional repressive activities of the tamoxifen-liganded ER.

The reasons for these discrepant results among different studies are not entirely clear. It is possible that the differences are due to the different model systems used. Because of the tissue-specific agonist/antagonist properties of tamoxifen, it is conceivable that the agonist activity of tamoxifen-bound ER in cell lines from various tissue origins occurs via a different mechanism than the agonist activity that results in tamoxifen-stimulated growth in breast cancer cells. For example, this may explain why tamoxifen-liganded ER becomes agonist in fibroblasts from N-CoR<sup>-/-</sup> mouse embryos (24). It is also conceivable that the results obtained in transcription experiments using transient expression of artificially high levels of corepressor that may not be biologically relevant are misleading. Another possibility is that the ER target genes that are regulated by N-CoR were not investigated in this study, which focused on the pS2 and vitellogenin genes, although we did examine the effects of N-CoR on cell proliferation, the net result of changes in gene expression. N-CoR was found on the promoters of ER-regulated genes by ChIP assay in another study (25, 26). In these ChIP experiments, researchers identified the corepressor on the promoters but did not examine function. If histone deacetylase activity is associated with tamoxifen-bound ER via an N-CoR corepressor complex, then loss of N-CoR function should increase the agonist activity of the tamoxifen-bound ER as well as the histone acetylation on ER-responsive genes. However, we did not observe increased transcriptional activity or acetylation with loss of N-CoR function via expression of the dominant-negative construct.

As the results described in this study suggest, it is possible that a corepressor, other than N-CoR, modulates tamoxifen-bound ER in breast cancer cells as stated previously. The corepressor SMRT is not a likely candidate because the N-CoR dominant-negative mutant used in this study would also be expected to block binding of SMRT to tamoxifen-bound ER due to the similar receptor-corepressor interaction domains of both N-CoR and SMRT (18, 22, 46, 47). The recent finding that various peptides, not including N-CoR or SMRT, containing the consensus CoRNR motif

can specifically interact with tamoxifen-liganded ER supports this idea (27).

In conclusion, the breast cancer cells in this study provide a useful biological system with which to study the agonist transcriptional and growth-stimulatory effects of tamoxifen that may occur as breast cancer patients become resistant to the drug. Surprisingly, our cumulative results suggest that expression of a dominant-negative N-CoR corepressor, which is sufficient to alter RAR-mediated transcription, does not affect tamoxifen-inhibited transcription or contribute to the acquisition of tamoxifen-stimulated growth in these ER-positive breast cancer cells.

### Experimental Animals

All animal experimentation described in this manuscript was conducted in accordance with mandated standards of humane care as described in the NIH Guide for the Care and Use of Laboratory Animals.

## MATERIALS AND METHODS

### GST Pull-Down Assay

The GST-ER $\alpha$  (DEF domains) and GST-RAR $\alpha$  were constructed as described previously (19, 48). <sup>35</sup>S-methionine-labeled IVT full-length N-CoR and dominant-negative N-CoR were incubated with GST fusion proteins bound to glutathione sepharose for 2 h at 4 C with the following hormone treatments: vehicle alone, estradiol (10<sup>-7</sup> M), 4-hydroxytamoxifen (10<sup>-6</sup> M), 9-*cis*-retinoic acid (10<sup>-6</sup> M), or all-*trans*-retinoic acid (10<sup>-6</sup> M). After washing of sepharose beads, samples were electrophoresed on SDS-PAGE gels and exposed to film.

### Transient Transfection Assays

MCF7D cells were plated in medium containing 5% charcoal-stripped fetal bovine serum. After 48 h cells were transfected with 100 ng of pCMV- $\beta$ -galactosidase, 750 ng of either vitellogenin-luciferase (49), pS2-luciferase, or RAR $\beta$ -luciferase (50) reporter constructs, and the indicated amounts of either vector alone, full-length N-CoR (19), or the dominant-negative N-CoR (30). After the transfections the cells were treated with vehicle alone, 9-*cis*-retinoic acid (10<sup>-6</sup> M), estradiol (10<sup>-9</sup> M), and/or 4-hydroxytamoxifen (10<sup>-7</sup> M) for a period of 16–24 h. The cells were harvested and assayed for luciferase expression. All transfections were done in triplicate and normalized for  $\beta$ -gal expression. The pS2-luciferase plasmid was constructed by removing the pS2 promoter from the pS2-chloramphenicol acetyl transferase (31) vector and inserting it into the pGL3-Basic vector (Promega Corp., Madison, WI).

### Construction of Inducible and Constitutive Stable Cell Lines

The Flag-tagged dominant-negative pcDNA3-N-CoR (dominant-negative N-CoR) containing amino acids 1586–2211 (19) was digested with *Hind*III and *Not*I to remove the N-CoR construct before ligation into a modified pIRES1hyg vector (CLONTECH, Palo Alto, CA) that contains the restriction sites *Eco*RV and *Not*I in the polylinker region. Individual

clones were expanded after transfection of the pIRES-DN N-CoR plasmid or empty vector into MCF7D cells. Western analysis using antibodies against the Flag epitope confirmed constitutive expression of the pIRES-DN N-CoR construct in the cells.

The Dimerizer Inducible System (Ariad Pharmaceuticals) was established by digesting the dominant-negative pcDNA3-N-CoR construct previously mentioned with *Hind*III and *Not*I and ligating it into a modified pLH-Z<sub>12</sub>-I-PL vector containing the same sites in the polylinker region. This plasmid or empty vector was transfected into a MCF7D cell line containing the other components of the inducible system. Several clones were expanded, and inducible expression of the dominant-negative construct was confirmed in Western analysis using antibodies that recognize the Flag epitope after overnight incubation with media containing vehicle or 50 nM inducer (AP1510).

### RT-PCR Analysis

The N-CoR dominant-negative or control cells were plated into medium containing 5% charcoal-stripped fetal bovine serum for 48 h before treatment. The cells were then treated with vehicle, estradiol (10<sup>-9</sup> M), 4-hydroxytamoxifen (10<sup>-7</sup> M), or 4-hydroxytamoxifen (10<sup>-7</sup> M) plus trichostatin A (100 nM) for 16–24 h. The RNA was harvested from the cells and RT-PCR analysis was performed using primers designed to amplify the region of pS2 mRNA from +54 to +268.

### ChIP Assays

The ChIP protocol was performed as previously described (51, 52). Briefly, N-CoR dominant-negative or control cells were treated with vehicle alone, estrogen, or 4-hydroxytamoxifen as described in *Transient Transfection Assays*. Before lysis, the cells were cross-linked with formaldehyde and the lysate was sonicated to produce chromatin fragments averaging 500 bp of DNA in length. The lysate was immunoprecipitated with antibodies specific for the acetylated forms of histone H3 and H4 (Upstate Biotechnology, Inc., Lake Placid, NY). The cross-linking was reversed by 4–5 h of heating at 65 C and the DNA was purified from each sample. The purified DNA was then used as a template in PCRs. Primers used in the PCRs were designed to amplify the regions of the pS2 promoter containing the nucleosome positioned at the TATA box (–19 to –216) and the nucleosome positioned at the ERE (–278 to –446).

### Cell Cycle and *in Vitro* Proliferation Experiments

The cell cycle and *in vitro* proliferation experiments were performed on the dominant-negative N-CoR or control cells plated in medium containing 5% charcoal-stripped fetal bovine serum for 48 h before treatment with vehicle alone, estrogen, or 4-hydroxytamoxifen as described in *Transient Transfection Assays*. For the cell cycle analyses, the cells were treated for 48 h and then harvested for DNA content analysis using flow cytometry. The *in vitro* proliferation assays were performed using the MTS assay, CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega Corp.), on the cells after 6 d of treatment. The assay is composed of two solutions, 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) and an electron-coupling reagent phenazine methosulfate. The MTS solution is reduced into formazan by dehydrogenase enzymes in metabolically active cells. Formazan is soluble in media and can be quantitated by obtaining the absorbance of the solution at 490 nm.

### In Vivo Proliferation Experiments

The constitutive dominant-negative N-CoR or control MCF7D cells were used in the nude mouse model of tamoxifen resistance previously described (43). Cells were injected sc, and an estrogen pellet was implanted in the ovariectomized mice to induce tumor growth. After 15 d of tumor growth, the pellet was removed and the mice were put on tamoxifen treatment for approximately 3 months. The tumors were harvested and pooled together to perform a Western analysis to confirm expression of the Flag-tagged dominant-negative N-CoR construct.

### Acknowledgments

We thank Gary C. Chamness for critical review and comment on the manuscript. We also acknowledge Mats Soderstrom and Christopher Glass for the gift of the N-CoR dominant-negative construct.

This work was supported by National Cancer Institute Spore Grant P50 CA-58183-06 from the National Institutes of Health.

Received July 13, 2001. Accepted April 23, 2003.

Address all correspondence and requests for reprints to: C. Kent Osborne, M.D., Baylor College of Medicine, The Breast Center, One Baylor Plaza, MS 600, Houston, Texas 77030. E-mail: kosborne@breastcenter.tmc.edu.

### REFERENCES

- Evans RM 1988 The steroid and thyroid hormone receptor superfamily. *Science* 240:889–895
- Knight WAD, Osborne CK, Yochmowitz MG, McGuire WL 1980 Steroid hormone receptors in the management of human breast cancer. *Ann Clin Res* 12:202–207
- Osborne CK, Yochmowitz MG, Knight WAD, McGuire WL 1980 The value of estrogen and progesterone receptors in the treatment of breast cancer. *Cancer* 46:2884–2888
- Saez RA, Osborne CK 1989 Hormonal treatment of advanced breast cancer. In: Kennedy BJ, ed. *Current clinical oncology*. New York: Alan R. Liss, Inc.; 163–172
- Grese TA, Sluka JP, Bryant HU, Cullinan GJ, Glasebrook AL, Jones CD, Matsumoto K, Palkowitz AD, Sato M, Termine JD, Winter MA, Yang NN, Dodge JA 1997 Molecular determinants of tissue selectivity in estrogen receptor modulators. *Proc Natl Acad Sci USA* 94:14105–14110
- Pham TA, Elliston JF, Nawaz Z, McDonnell DP, Tsai MJ, O'Malley BW 1991 Antiestrogen can establish nonproductive receptor complexes and alter chromatin structure at target enhancers. *Proc Natl Acad Sci USA* 88:3125–3129
- Osborne CK, Fuqua SA 1994 Mechanisms of tamoxifen resistance. *Breast Cancer Res Treat* 32:49–55
- Heery DM, Kalkhoven E, Hoare S, Parker MG 1997 A signature motif in transcriptional co-activators mediates binding to nuclear receptors. *Nature* 387:733–736
- Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. *Cell* 95:927–937
- Onate SA, Tsai SY, Tsai MJ, O'Malley BW 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* 270:1354–1357
- Hong H, Kohli K, Trivedi A, Johnson DL, Stallcup MR 1996 GRIP1, a novel mouse protein that serves as a transcriptional coactivator in yeast for the hormone binding domains of steroid receptors. *Proc Natl Acad Sci USA* 93:4948–4952
- Voegel JJ, Heine MJ, Zechel C, Chambon P, Gronemeyer H 1996 TIF2, a 160 kDa transcriptional mediator for the ligand-dependent activation function AF-2 of nuclear receptors. *EMBO J* 15:3667–3675
- Hanstein B, Eckner R, DiRenzo J, Halachmi S, Liu H, Searcy B, Kurokawa R, Brown M 1996 p300 is a component of an estrogen receptor coactivator complex. *Proc Natl Acad Sci USA* 93:11540–11545
- Anzick SL, Kononen J, Walker RL, Azorsa DO, Tanner MM, Guan XY, Sauter G, Kallioniemi OP, Trent JM, Meltzer PS 1997 AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science* 277:965–968
- Torchia J, Rose DW, Inostroza J, Kamei Y, Westin S, Glass CK, Rosenfeld MG 1997 The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. *Nature* 387:677–684
- Li H, Gomes PJ, Chen JD 1997 RAC3, a steroid/nuclear receptor-associated coactivator that is related to SRC-1 and TIF2. *Proc Natl Acad Sci USA* 94:8479–8484
- Chen H, Lin RJ, Schiltz RL, Chakravarti D, Nash A, Nagy L, Privalsky ML, Nakatani Y, Evans RM 1997 Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* 90:569–580
- Jackson TA, Richer JK, Bain DL, Takimoto GS, Tung L, Horwitz KB 1997 The partial agonist activity of antagonist-occupied steroid receptors is controlled by a novel hinge domain-binding coactivator L7/SPA and the corepressors N-CoR or SMRT. *Mol Endocrinol* 11:693–705
- Horlein AJ, Naar AM, Heinzl T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, Rosenfeld MG 1995 Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* 377:397–404
- Chen JD, Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature* 377:454–457
- Smith CL, Nawaz Z, O'Malley BW 1997 Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. *Mol Endocrinol* 11:657–666
- Zhang X, Jeyakumar M, Petukhov S, Bagchi MK 1998 A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. *Mol Endocrinol* 12:513–524
- Lavinsky RM, Jepsen K, Heinzl T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW 1998 Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci USA* 95:2920–2925
- Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW, Rosenfeld MG 2000 Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell* 102:753–763
- Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. *Cell* 103:843–852
- Shang Y, Brown M 2002 Molecular determinants for the tissue specificity of SERMs. *Science* 295:2465–2468
- Huang HJ, Norris JD, McDonnell DP 2002 Identification of a negative regulatory surface within estrogen receptor  $\alpha$  provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Mol Endocrinol* 16:1778–1792
- Laherty CD, Billin AN, Lavinsky RM, Yochum GS, Bush AC, Sun JM, Mullen TM, Davie JR, Rose DW, Glass CK, Rosenfeld MG, Ayer DE, Eisenman RN 1998 SAP30, a

- component of the mSin3 corepressor complex involved in N-CoR-mediated repression by specific transcription factors. *Mol Cell* 2:33–42
29. Osborne CK, Coronado EB, Robinson JP 1987 Human breast cancer in the athymic nude mouse: cytostatic effects of long-term antiestrogen therapy. *Eur J Cancer Clin Oncol* 23:1189–1196
  30. Soderstrom M, Vo A, Heinzel T, Lavinsky RM, Yang WM, Seto E, Peterson DA, Rosenfeld MG, Glass CK 1997 Differential effects of nuclear receptor corepressor (N-CoR) expression levels on retinoic acid receptor-mediated repression support the existence of dynamically regulated corepressor complexes. *Mol Endocrinol* 11:682–692
  31. Jakowlew SB, Breathnach R, Jeltsch JM, Masiakowski P, Chambon P 1984 Sequence of the pS2 mRNA induced by estrogen in the human breast cancer cell line MCF-7. *Nucleic Acids Res* 12:2861–2878
  32. Mizzen CA, Allis CD 1998 Linking histone acetylation to transcriptional regulation. *Cell Mol Life Sci* 54:6–20
  33. Walia H, Chen HY, Sun JM, Holth LT, Davie JR 1998 Histone acetylation is required to maintain the unfolded nucleosome structure associated with transcribing DNA. *J Biol Chem* 273:14516–14522
  34. Nightingale KP, Wellinger RE, Sogo JM, Becker PB 1998 Histone acetylation facilitates RNA polymerase II transcription of the *Drosophila* hsp26 gene in chromatin. *EMBO J* 17:2865–2876
  35. Pazin MJ, Kadonaga JT 1997 What's up and down with histone deacetylation and transcription? *Cell* 89:325–328
  36. Alland L, Muhle R, Hou Jr H, Potes J, Chin L, Schreiber-Agus N, DePinho RA 1997 Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. *Nature* 387:49–55
  37. Heinzel T, Lavinsky RM, Mullen TM, Soderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK, Rosenfeld MG 1997 A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression. *Nature* 387:43–48
  38. Hassig CA, Fleischer TC, Billin AN, Schreiber SL, Ayer DE 1997 Histone deacetylase activity is required for full transcriptional repression by mSin3A. *Cell* 89:341–347
  39. Kadosh D, Struhl K 1997 Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters. *Cell* 89:365–371
  40. Laherty CD, Yang WM, Sun JM, Davie JR, Seto E, Eisenman RN 1997 Histone deacetylases associated with the mSin3 corepressor mediate mad transcriptional repression. *Cell* 89:349–356
  41. Nagy L, Kao HY, Chakravarti D, Lin RJ, Hassig CA, Ayer DE, Schreiber SL, Evans RM 1997 Nuclear receptor repression mediated by a complex containing SMRT, mSin3A, and histone deacetylase. *Cell* 89:373–380
  42. Sewack GF, Hansen U 1997 Nucleosome positioning and transcription-associated chromatin alterations on the human estrogen-responsive pS2 promoter. *J Biol Chem* 272:31118–31129
  43. Osborne CK, Coronado E, Allred DC, Wiebe V, DeGregorio M 1991 Acquired tamoxifen resistance: correlation with reduced breast tumor levels of tamoxifen and isomerization of trans-4-hydroxytamoxifen. *J Natl Cancer Inst* 83:1477–1482
  44. Osborne CK, Bardou V, Hilsenbeck SG, Fuqua SA, Hopp TA, Chamness GC, Wong J, Schiff R, Allred DC, Clark GM, The estrogen receptor coactivator AIB1(SRC3) in combination with HER-2 is a prognostic and predictive marker in patients with breast cancer. Proc American Society of Clinical Oncology Annual Conference, Orlando, FL, 2002
  45. Osborne CK, Bardou V, Hopp TA, Chamness GC, Hilsenbeck SG, Fuqua SA, Wong J, Allred DC, Clark GM, Schiff R 2003 Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst* 95:353–361
  46. Li H, Leo C, Schroen DJ, Chen JD 1997 Characterization of receptor interaction and transcriptional repression by the corepressor SMRT. *Mol Endocrinol* 11:2025–2037
  47. Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, Cobb JE, McKee DD, Galardi CM, Plunket KD, Nolte RT, Parks DJ, Moore JT, Klier SA, Willson TM, Stimmel JB 2002 Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPAR $\alpha$ . *Nature* 415:813–817
  48. Zhao HH, Herrera RE, Coronado-Heinsohn E, Yang MC, Ludes-Myers JH, Seybold-Tilson KJ, Nawaz Z, Yee D, Barr FG, Diab SG, Brown PH 2001 Forkhead homologue in rhabdomyosarcoma functions as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions. *J Biol Chem* 276:27907–27912
  49. Burch JB, Evans MI, Friedman TM, O'Malley PJ 1988 Two functional estrogen response elements are located upstream of the major chicken vitellogenin gene. *Mol Cell Biol* 8:1123–1131
  50. Yu VC, Delsert C, Andersen B, Holloway JM, Devary OV, Naar AM, Kim SY, Boutin JM, Glass CK, Rosenfeld MG 1991 RXR  $\beta$ : a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. *Cell* 67:1251–1266
  51. Dedon PC, Soultis JA, Allis CD, Gorovsky MA 1991 A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. *Anal Biochem* 197:83–90
  52. Orlando V, Strutt H, Paro R 1997 Analysis of chromatin structure by *in vivo* formaldehyde cross-linking. *Methods* 11:205–214

