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13. ABSTRACT ***(Maximum 200 Words)***

The major goal of this research is to study the mechanism of corepressor proteins N-CoR in normal and breast cancer cells. It has been shown that N-CoR is capable of association with antagonist-bound estrogen receptor in the hormone-dependent human breast cancer (Shang et al., 2000). In addition, it has been shown that in the tamoxifen resistant breast cancer cells N-CoR expression level is decreased (Lavinsky et al, 1998). These observations highlight N-CoR as key regulators of breast cancer pharmacology. During the course of our research sponsored the current Postdoctoral Traineeship, we investigated N-CoR functions by identification of new N-CoR binding proteins and new mechanism that regulate N-CoR activities. The major findings are summarized as follows. First, we discovered that N-CoR physically interacts with B-Myb, a key cell cycle regulator. The significance of these B-Myb-corepressor interactions was confirmed by the finding that B-Myb mutants, which were unable to bind N-CoR, exhibited constitutive transcriptional activity. We also have determined that phosphorylation by cdk2/cyclin A blocks the interaction between B-Myb and N-CoR and that mutation of the corepressor binding site within B-Myb bypasses the requirement for this phosphorylation event. In our second finding, we discovered that corepressor N-CoR directly interacts with coactivator ACTR. Interestingly, ACTR is over-amplified in a number of breast cancer cells (Anzick et al., 1997). We observed that N-CoR contributes to transcriptional activation by recruiting ACTR to nuclear receptors prior to ligand activation. Our results suggested that transcriptional repression and activation, the two processes which both involved in breast cancer biology, are integrated in a manner that are not previously anticipated.

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Introduction

The major goal of this research is to study the mechanism of corepressor proteins in normal and breast cancer cells. It has been shown that N-CoR is capable of association with antagonist-bound estrogen receptor in the hormone-dependent human breast cancer (1, 2). The expression level of N-CoR was significantly decreased in the tamoxifen resistant MCF-7 breast cancer cells compared to the tamoxifen-sensitive counterparts (3). These findings strengthened the pharmacological significance of N-CoR in breast cancer cells. In this study we have identified novel functions of N-CoR by which N-CoR physically interacts with (a) cell cycle related transcription factor B-Myb and (b) nuclear receptor coactivator ACTR.

The B-Myb transcription factor has been implicated in coordinating the expression of genes involved in cell-cycle regulation (4, 5). Although it is expressed in a ubiquitous manner, its transcriptional activity is repressed until the G1-S phase of the cell cycle by an unknown mechanism. We used biochemical and cell based assays to demonstrate that the nuclear receptor corepressors, N-CoR and SMRT, interact with B-Myb. The significance of these B-Myb-corepressor interactions was confirmed by the finding that B-Myb mutants, which were unable to bind N-CoR, exhibited constitutive transcriptional activity. It has been shown previously that phosphorylation of B-Myb by cdk2/cyclin A enhances its transcriptional activity (6). We have now determined that phosphorylation by cdk2/cyclin A blocks the interaction between B-Myb and N-CoR and that mutation of the corepressor binding site within B-Myb bypasses the requirement for this phosphorylation event. Cumulatively, these findings suggest that the nuclear corepressors N-CoR and SMRT serve a previously unappreciated role as regulators of B-Myb, a proto-oncoprotein participated in cell cycle progression. Thus, N-CoR and SMRT could play roles in maintaining normal cell cycles and prevent abnormality of cells cycle that has been frequently observed in cancer cells.

ACTR is a coactivator protein for multiple nuclear receptors. The roles of ACTR in breast cells were highlighted by the finding that in the ER positive breast and ovarian cancer cells, the expression level of coactivator ACTR is significantly increased (7). We have undertaken a phage display approach to identify the protein-protein interaction surfaces on N-

CoR in order to define the biochemical processes that modulate PR transcriptional activity. Among the phage peptides what associate N-CoR, one peptide shared striking similarity to ACTR. This finding has subsequently lead to the discovery that corepressor N-CoR directly interacts with coactivator ACTR. We observed that N-CoR contributes to transcriptional activation by recruiting ACTR to nuclear receptors prior to ligand activation. These findings suggested that transcriptional repression and activation, the two processes which both involved in breast cancer biology, are integrated in a manner that are not previously anticipated.

Body

1. The transcription factor B-Myb is maintained in an inhibited state in target cells through its interaction with the nuclear corepressors N-COR and SMRT.
The specific research is described in Appendix 1 (Li and McDonnell, 2002).

2. Direct interactions between corepressors and coactivators permit the integration of nuclear receptor-mediated repression and activation.
The specific research is described in Appendix 2 (Li et al., 2002).

Key Research Accomplishments

1. Identification of direct interaction between B-Myb and corepressors N-CoR and SMRT.

- B-Myb interacts directly with the nuclear receptor corepressors N-CoR and SMRT.
- N-CoR and SMRT act through the previously defined negative regulatory domain of B-Myb.
- N-CoR and SMRT function as repressors of B-Myb transcriptional activity.
- Cdk2/cyclin A mediated phosphorylation blocks the ability of B-Myb to interact with corepressors.
- CBP interacts with and potentiates B-Myb transcriptional activity.

2. Identification of direct interaction between corepressor N-CoR and coactivator ACTR.

- Discovered that N-CoR interacts directly with ACTR in vitro and in vivo.
- Characterization of the interaction surface on N-CoR and ACTR.
- Identified that N-CoR, ACTR and TR form a trimeric complex in target cells.
- Identified that N-CoR potentiates transcriptional activation of TR/ACTR complex.
- Identified that ACTR reverses N-CoR-mediated transcriptional repression.

Reportable Outcomes

Journal publications:

Li, X. and McDonnell, D.P. (2002) The transcription factor B-Myb is maintained in an inhibited state in target cells through its interaction with the nuclear corepressors N-CoR and SMRT. Mol. Cell. Biol. 22: 3663-3673.

Li, X., Kimbrel, E.A., Kenan D.J, and McDonnell, D.P. (2002) Direct interactions between corepressors and coactivators permit the integration of nuclear receptor mediated repression and activation. Mol. Endocrinol. In press.

Conclusions

N-CoR is a corepressor protein that is widely expressed in a variety of cell types and is utilized by multiple signaling pathways. In the research work sponsored by this Postdoctoral Traineeship, we investigated novel mechanisms that regulate the functions of N-CoR in the normal and breast cancer cells. We found that the nuclear corepressors N-CoR and SMRT serve a previously unappreciated role as regulators of B-Myb, a proto-oncoprotein participated in cell cycle progression. N-CoR and SMRT could play roles in maintaining normal cell cycles and prevent abnormality of cells cycle that has been frequently observed in cancer cells. In addition, we found that that the corepressor N-CoR directly interacts with coactivator ACTR. We observed that N-CoR contributes to transcriptional activation by recruiting ACTR to nuclear receptors prior to ligand activation. These findings suggested that transcriptional repression and activation, the two processes which both involved in breast cancer biology, are integrated in a manner that are not previously anticipated.

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APPENDIX 1

MOLECULAR AND CELLULAR BIOLOGY, June 2002, p. 3663–3673 Vol. 22, No. 11
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The Transcription Factor B-Myb Is Maintained in an Inhibited State in Target Cells through Its Interaction with the Nuclear Corepressors N-CoR and SMRT

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The B-Myb transcription factor has been implicated in coordinating the expression of genes involved in cell cycle regulation. Although it is expressed in a ubiquitous manner, its transcriptional activity is repressed until the G_1-S phase of the cell cycle by an unknown mechanism. In this study we used biochemical and cell-based assays to demonstrate that the nuclear receptor corepressors N-CoR and SMRT interact with B-Myb. The significance of these B-Myb–corepressor interactions was confirmed by the finding that B-Myb mutants, which were unable to bind N-CoR, exhibited constitutive transcriptional activity. It has been shown previously that phosphorylation of B-Myb by cdk2/cyclin A enhances its transcriptional activity. We have now determined that phosphorylation by cdk2/cyclin A blocks the interaction between B-Myb and N-CoR and that mutation of the corepressor binding site within B-Myb bypasses the requirement for this phosphorylation event. Cumulatively, these findings suggest that the nuclear corepressors N-CoR and SMRT serve a previously unappreciated role as regulators of B-Myb transcriptional activity.

The transcription factor B-Myb is a member of the family of proteins encoded by the *myb* proto-oncogenes, which also includes the structurally related proteins A-Myb and c-Myb (26, 29, 31). These three transcription factors, while functionally distinct, have been grouped based on amino acid sequence homology among family members. Unlike A-Myb and c-Myb, the transcriptional activity of B-Myb is constitutively suppressed and appears to be manifest only at specific stages during the cell cycle, during which it is involved in the regulation of a number of genes generally associated with cell proliferation, including *cdc2*, c-myc, and those encoding DNA polymerase alpha and B-Myb itself (24, 27, 28, 36). More compelling evidence in support of a specific role for B-Myb in proliferation comes from studies which have demonstrated that ablation of B-Myb by antisense oligonucleotides inhibits the growth of human hematopoietic cells and glioblastomas (2, 22), while constitutive expression of B-Myb overrides p53-induced and interleukin-6-induced cell cycle arrest (7, 21). These functional characteristics distinguish B-Myb from c-Myb and A-Myb and suggest that these three Myb proteins have nonredundant functions in gene regulation.

Under most circumstances, B-Myb is unable to activate transcription of its cognate target genes and indeed it may even repress the basal level of transcription of the genes with which it associates (1, 19, 23, 28, 37). However, in proliferating cells it has been observed that as cells progress from the G_1 phase to the S phase of the cell cycle, there is an increase in both the expression level and the transcriptional activity of B-Myb. The enhanced expression level can likely be explained by the fact that the B-Myb promoter contains a functional E2F binding site (18). The G_1-S phase-restricted manifestation of B-Myb transcriptional activity appears to require the activity of cdk2/cyclin A (1, 19, 28, 37). Direct phosphorylation of B-Myb by cdk2/cyclin A has been demonstrated, though it is not clear how this modification actually enables B-Myb transcriptional activity. It has been suggested that phosphorylation is required in order to overcome an inhibitory function contained within the carboxyl terminus of the receptor, as deletion of this region of the protein enables B-Myb transcriptional activity in the absence of phosphorylation (19, 36). It has not been determined whether the inhibitory activity of the carboxyl terminus of B-Myb is mediated by an autoinhibitory intramolecular interaction or if it requires an intermolecular association with a corepressor protein.

Many transcription factors require an activating event such as ligand binding or phosphorylation to enable them to manifest transcriptional activity. However, B-Myb distinguishes itself from most transcription factors in that its transcriptional activity appears to be actively suppressed. Moreover, in the absence of an activating event this transcription factor may suppress the basal transcription of target genes. Thus, it is not clear if B-Myb activation merely requires it in order to overcome repression or if a second event, subsequent to relief of repression, is required in order to permit it to manifest transcription activity. In looking for insights into this issue, we noticed the similarity between the proposed mechanisms of action of B-Myb and of the nuclear receptor for thyroid hormone (TR). Specifically, it has been shown that TR resides on the promoters of target genes in the absence of the hormone and is able to repress transcription by bringing to target genes the nuclear receptor corepressors N-CoR and SMRT (9, 14). Upon activation by ligand, the corepressors are displaced, coactivators are recruited, and TR-mediated transcriptional activation is permitted. Based on the similarity of the mechanisms of action of TR and B-Myb, we hypothesize that the

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nuclear receptor corepressors N-CoR and SMRT may be involved in B-Myb action (9, 14). N-CoR and SMRT are homologous corepressors that can associate with the unliganded TR and the retinoic acid receptor, enabling these receptors to repress the basal transcriptional activity of their respective target genes. Within these proteins, specific repression domains that function by recruiting class I and II histone deacetylases (HDACs) to target gene promoters have been mapped (13). When associated with DNA, this complex can deacetylate chromatin and silence transcription. In addition to their ability to interact with nuclear receptors, N-CoR and SMRT have also been shown to interact with and regulate the transcriptional activities of a variety of unrelated transcription factors, including Hox, MyoD, MAD, and SHARP (3, 4, 13, 33). Thus, the influence of these corepressors appears to be more universal than originally anticipated. Consequently, we have investigated whether the corepressors N-CoR and SMRT have roles in regulating the transcriptional activity of B-Myb.

MATERIALS AND METHODS

Plasmids. The expression plasmids of murine B-Myb pcDNA3-B-Myb and pcDNA3–B-Myb$_{1-561}$ were provided by R. Watson (Imperial College School of Medicine, London, United Kingdom). The plasmid pCMX–N-CoR was from M. G. Rosenfeld (University of California, San Diego). The plasmid pCMX-Gal4–C′SMRT was from J. D. Chen (University of Massachusetts). Plasmids pCMV-cdk2 and pCMV-cdk2DN were from E. Harlow (Massachusetts General Hospital). The plasmid pCMV-cyclinA was from J. R. Nevins (Duke University). The plasmids of pR3SV and pRMb3SV–c-Myb were from T. P. Bender (University of Virginia). Gal4′–C′N-CoR was generated by PCR of a DNA fragment corresponding to N-CoR residues 1944 to 2453 which were then cloned into a PM vector (Clontech). Plasmids Myc–N-CoR and Myc–N-CoR$_{759-2453}$ were generated by subcloning DNA fragments from pCMX–N-CoR into a pcDNA3-5myc vector. The plasmid for GST fusion of N-CoR ID1 domain (amino acids [aa] 2063 to 2142) was generated by PCR of the corresponding region of N-CoR which were then subcloned into a pGEX-6P-1 vector (Pharmacia). Series of VP16–B-Myb plasmids were made by PCR of the corresponding DNA fragments which were then subcloned into the VP16 vector (Clontech). The reporter plasmid 3A-TK-luc was generated by annealing a pair of 69-mer oligonucleotides containing three copies of Myb binding site A and ligating them into a TK-Luc vector by using *Hin*dIII and *Bam*HI sites. The 3A oligonucleotides were as follows: forward, AGCTCTAAAAAACCGTTATAATGTACACTAAAAAAC CGTTATAATGTACTCTAAAAAACCGTTATAATG; reverse, GATTTTTT GGCAATATTACATGTGATTTTTTGGCAATATTACATGAGATTTTTTG GCAATATTACCTAG.

GST pull-down assay. Glutathione *S*-transferase (GST) fusion proteins were expressed in bacterial strain BL21 and were isolated by glutathione-conjugated Sepharose 4B beads (Pharmacia). Proteins incorporating [^{35}S]methionine ([^{35}S]Met) were generated by the TNT kit (Promega). GST fusion proteins and beads were incubated with [^{35}S]Met-labeled protein in NETN buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM NaCl, and 0.5% NP-40) for 16 h at 4°C. Bound proteins were washed twice with NETN buffer and twice with buffer A (2 mM Tris-HCl [pH 7.4], 0.5 mM EDTA, 0.5% NP-40) and were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography.

Cell culture and transfection. All cultured cells were maintained in minimum essential medium (Life Technologies) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Culture dishes were precoated with 0.1% gelatin for 10 min at 25°C. Cells were grown at 37°C in 5% CO_2. Protocols for transient transfection and luciferase assays were essentially as previously described (8). Briefly, cells were split among 10-mm-diameter culture dishes (for coimmunoprecipitation [co-IP]) and 24-well plates (for luciferase assay) 1 day before the transfection. The lipid-mediated transient transfection was performed with a mixture of Lipofectin (Life Technologies) and plasmid DNA containing 3 μg of DNA for a triplicate of luciferase assay in a 24-well plate (Corning Incorporated) or 18 μg of DNA for a 10-mm-diameter dish (Falcon). Cells were incubated with the Lipofectin-DNA mixture for 3 to 7 h and were then incubated in normal media for an additional 24 to 48 h. For the luciferase assays, luciferase readings were normalized using signals of β-galactosidase (β-Gal) and the final results were shown as means ± standard deviations of triplicate measurements. All data shown are representative of at least three experiments.

Immunoprecipitations and Western blots. Cultured cells were washed with phosphate-buffered saline and lysed with buffer T containing 20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, and protease inhibitors (Roche Molecular Biochemicals) for 30 min on ice. The whole-cell lysates were clarified by centrifugation and were then precleared by protein A-Sepharose CL-4B (Amersham Biosciences) for 1 h at 4°C. Antibody was then mixed with lysates for 2 h at 25°C or overnight at 4°C. Protein A-Sepharose was added for 2 h and then washed with buffer T for 30 min. Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a Hybond-C nitrocellulose membrane (Amersham Biosciences). The membrane was blocked with a buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 5% nonfat dried milk for 1 h. Primary antibody (1 to 3 μg) was diluted in phosphate-buffered saline plus 0.1% Tween 20 and was incubated with the membrane for 2 h at 25°C or overnight at 4°C. Subsequently the secondary antibodies (diluted 1 to 4,000) were incubated with the membrane for 1 h at 25°C. Anti-B-Myb rabbit polyclonal (N-19), anti-N-CoR goat polyclonal (C-20), anti-myc mouse monoclonal (9E10), anti-myc rabbit polyclonal (A14), and anti TRβ1 (J51) mouse monoclonal antibodies were from Santa Cruz Biotechnology.

RESULTS

B-Myb interacts directly with the nuclear receptor corepressors N-CoR and SMRT. The transcriptional activator B-Myb is maintained in an inactive state in target cells presumably as a consequence of its ability to interact with proteins that function as transcriptional corepressors (26, 29, 31). Based on the general similarity in the proposed mechanism of action of B-Myb and that described for the nuclear hormone receptors, we hypothesized that the corepressors N-CoR and SMRT might be involved in maintaining this transcription factor in a transcriptionally inactive state. Accordingly, we utilized a mammalian two-hybrid assay to examine whether N-CoR (or SMRT) and B-Myb are capable of interacting in intact cells. We first tested the ability of a fusion protein comprising the C terminus of N-CoR (aa 1944 to 2453) fused to the Gal4 DNA binding domain (Gal4–C′N-CoR) to tether a VP16–B-Myb fusion to a Gal4-responsive luciferase reporter. The N-CoR domain chosen does not contain the previously defined repressor domains but rather an intact receptor interaction domain (ID) (11, 14) (Fig. 1A). The results of this initial experiment indicated that Gal4–C′N-CoR is capable of interacting with VP16–B-Myb (Fig. 1B). We noticed that even in the absence of the Gal4–C′N-CoR fusion the VP16–B-Myb protein displayed a significant level of basal transcriptional activity. This may be the result of a nonspecific association between the DNA binding domain of B-Myb and the reporter. To avoid the confounding influence of the observed background activity, we created VP16–B-Myb$_{197-704}$, a mutant that lacks the previously defined B-Myb DNA binding domain. This mutant protein did not display significant basal transcriptional activity but maintained strong Gal4–C′N-CoR binding activity (Fig. 1C). A similar series of studies led to the observation that SMRT, a corepressor protein homologous to N-CoR, was also capable of interacting with B-Myb. Specifically, it was determined that Gal4-C′SMRT interacts with both VP16–B-Myb and VP16–B-Myb$_{197-704}$ (Fig. 1B and C). Thus, N-CoR and SMRT, two structurally related corepressors, are capable of interacting with B-Myb within intact cells.

We confirmed that B-Myb and N-CoR can interact by performing co-IP assays on protein extracts from cells transiently transfected with plasmids expressing B-Myb and myc-tagged

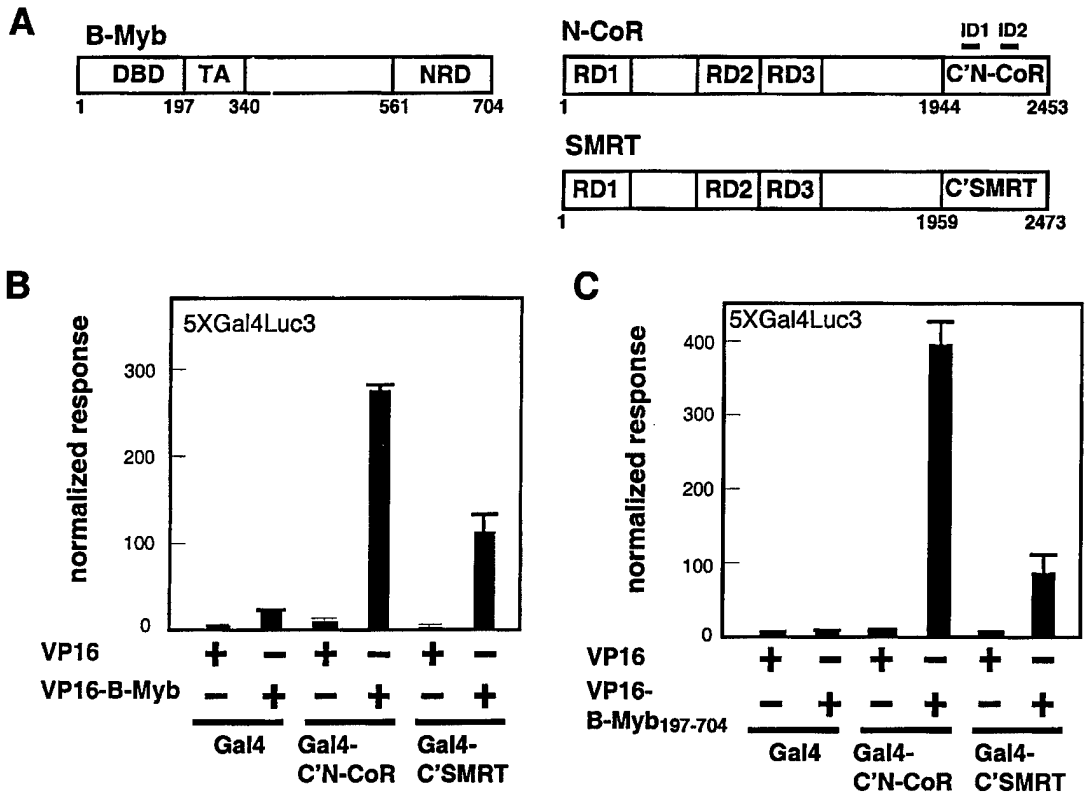

FIG. 1. B-Myb interacts with C'N-CoR and C'SMRT. (A) Schematic diagrams of N-CoR, SMRT, and B-Myb are adapted from previous reports (15, 31). (B) B-Myb interacts with C'N-CoR and C'SMRT in a mammalian two-hybrid assay. The interaction between GAL4 fusion and VP16 fusion was measured through a mammalian two-hybrid assay on the 5XGal4Luc3 reporter plasmid in CV1 cells. A cytomegalovirus β-galactosidase (CMV–β-Gal) internal control plasmid was used to normalize the luciferase values for transfection efficiency. Means ± standard deviations of triplicates are shown. Results are representative of three independent assays. (C) B-Myb$_{197-704}$ interacts with C'N-CoR and C'SMRT in the mammalian two-hybrid assay using the same assay conditions as those described for panel B. Results are representative of three independent assays.

N-CoR$_{759-2453}$. In these studies, proteins were immunoprecipitated from extracts with an anti-B-Myb antibody and the presence of N-CoR within the immunoprecipitates was evaluated by Western immunoblotting using an anti-myc antibody. The results indicated that N-CoR$_{759-2453}$ and B-Myb can form a complex inside cells (Fig. 2A). To further demonstrate that N-CoR and B-Myb interact in cells, we performed the co-IP in the reciprocal manner. In this experiment, myc–N-CoR$_{759-2453}$ was immunoprecipitated and the level of associated B-Myb was detected by using a Western blot. As shown in Fig. 2B, the results confirm that N-CoR and B-Myb can be coimmunoprecipitated from cells.

The corepressors N-CoR and SMRT were originally identified as proteins that interacted with and suppressed the basal activity of the thyroid and retinoic acid receptors (9, 14). The latter receptors have been shown by most approaches to exhibit robust interactions with SMRT and N-CoR in the absence of an activating ligand. To further characterize the B-Myb–corepressor interactions, we performed a comparative analysis of TR–N-CoR and B-Myb–N-CoR interactions by assessing the level of TRβ or B-Myb that could be immunoprecipitated with myc-tagged N-CoR from cell extracts. Notwithstanding the assumptions inherent in this type of assay, expression levels of the proteins, epitope accessibility, etc., it was clear that TRβ is a more avid N-CoR binder than B-Myb (Fig. 2B). What this actually means, however, is unclear since it has been shown by our group and others that the progesterone and estrogen receptors, two steroid hormone receptors whose pharmacology is modulated dramatically by N-CoR and SMRT, also demonstrate relatively weak corepressor interactions (35). We feel that the functional data (see below) and the observation that the endogenous proteins N-CoR and B-Myb can be coimmunoprecipitated from intact cells indicate that the interaction observed between N-CoR and B-Myb is physiologically relevant (Fig. 2C).

As a final step in evaluating the physical interaction between B-myb and N-CoR, we used in vitro GST pull-down assays to define the region(s) within N-CoR that mediate this interaction (Fig. 2D). Since C'N-CoR interacts with both B-Myb and nuclear receptors such as TR, we considered it possible that the two proteins interact with the same domain within N-CoR. Thus, we examined whether B-Myb can interact with the minimal nuclear receptor IDs of N-CoR. Specifically, the interaction of B-Myb with ID1 (aa 2063 to 2142), an 80-aa domain within C'N-CoR, was assessed, and it was found that this domain was indeed capable of interacting with B-Myb.

N-CoR and SMRT function as repressors of B-Myb tran-

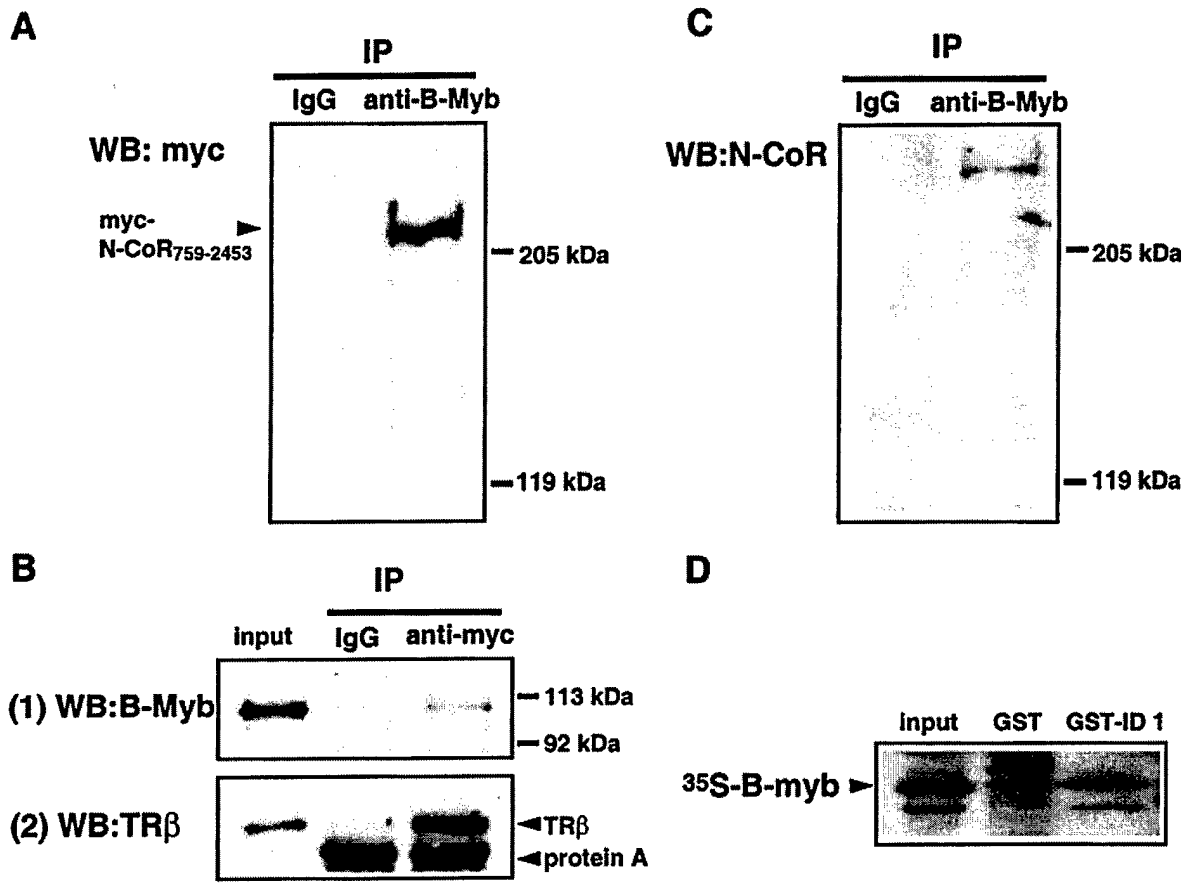

FIG. 2. B-Myb interacts with N-CoR and SMRT in cells. (A) Co-IP between B-Myb and N-CoR$_{759-2453}$. 293T cells were transfected with pcDNA3–B-Myb and pcDNA3-Myc–N-CoR$_{759-2453}$ plasmids and were grown for an additional 48 h. Whole-cell lysates (2 mg of total protein) were immunoprecipitated with rabbit anti-B-Myb and were then immunoblotted with mouse anti-myc. (B) 293T cells were transfected with pcDNA3-Myc–N-CoR$_{759-2453}$ plus pcDNA3–B-Myb plasmids (upper panel, labeled 1) or by pcDNA3-Myc–N-CoR$_{759-2453}$ plus pcDNA3-TRβ1 plasmids (lower panel, labeled 2). Equal molar amounts of pcDNA3–B-Myb and pcDNA2-TRβ1 were used in the transfection, and equal amounts of cell lysates were used in immunoprecipitation. Mouse anti-myc was used for panel 1 and rabbit anti-myc was used for panel 2 for immunoprecipitation. Input, 1%. (C) Co-IP between endogenous B-Myb and N-CoR. Whole-cell lysates of untransfected 293T cells were immunoprecipitated with rabbit anti-B-Myb and were then immunoblotted with goat anti-N-CoR. (D) B-Myb interacts with N-CoR domain ID1 (aa 2063 to 2142) in a GST pull-down assay. Two micrograms of GST or GST-ID1 were incubated with 10 μl of TNT lysates containing ^{35}S-labeled B-Myb. Protocols are as described in Materials and Methods. Input, 10%.

scriptional activity. The observation that B-Myb and N-CoR or SMRT interact raised the possibility that these corepressors may negatively regulate B-Myb transcriptional activity. If this were the case, then B-Myb transcriptional activity should be enhanced in cells in which the repression activity of endogenous N-CoR and SMRT is inhibited. To test this idea, we used the following three approaches to inhibit the activities of the two repressors and assessed the effects of these manipulations on B-Myb transcriptional activity: (i) the dominant negative inhibitors C′N-CoR or C′SMRT were expressed, (ii) unliganded TRβ was expressed as a competitive inhibitor of B-Myb–corepressor interactions, and (iii) trichostatin A (TSA) was used to inhibit the HDAC activity associated with N-CoR and SMRT. The transcriptional activity of B-Myb was assessed in transfected CV-1 cells by using a reporter plasmid, 3A-TK-luc, that contains three copies of the Myb binding site A from the mim-1 gene (25) (Fig. 3A). As expected, B-Myb expression alone was not sufficient to activate the 3A-TK-luc reporter. However, coexpression of C′N-CoR or C′SMRT did permit a significant increase in B-Myb transactivation activity (Fig. 3A). A similar result was achieved when we overexpressed TR (TRβ1) to sequester endogenous SMRT and N-CoR (Fig. 3B). It has been shown previously that unliganded TR forms a stable complex with the C terminus of N-CoR and SMRT and that the complex is dissociated when TR interacts with its cognate hormone T3 (9, 14). Not surprisingly, therefore, the ability of TR to enhance the transactivation activity of B-Myb was lost when T3 was added to the transfected cells (Fig. 3B). We believe that the slight enhancement of reporter activity observed in the presence of overexpressed C′N-CoR, C′SMRT, or TRβ1 in the absence of a B-Myb expression plasmid may represent an enhancement of the transcriptional activity of the endogenously expressed B-Myb protein.

N-CoR and SMRT repress transcriptional activity by recruiting HDACs to target gene promoters (13). If N-CoR and SMRT are bona fide repressors of B-Myb, as we propose, then

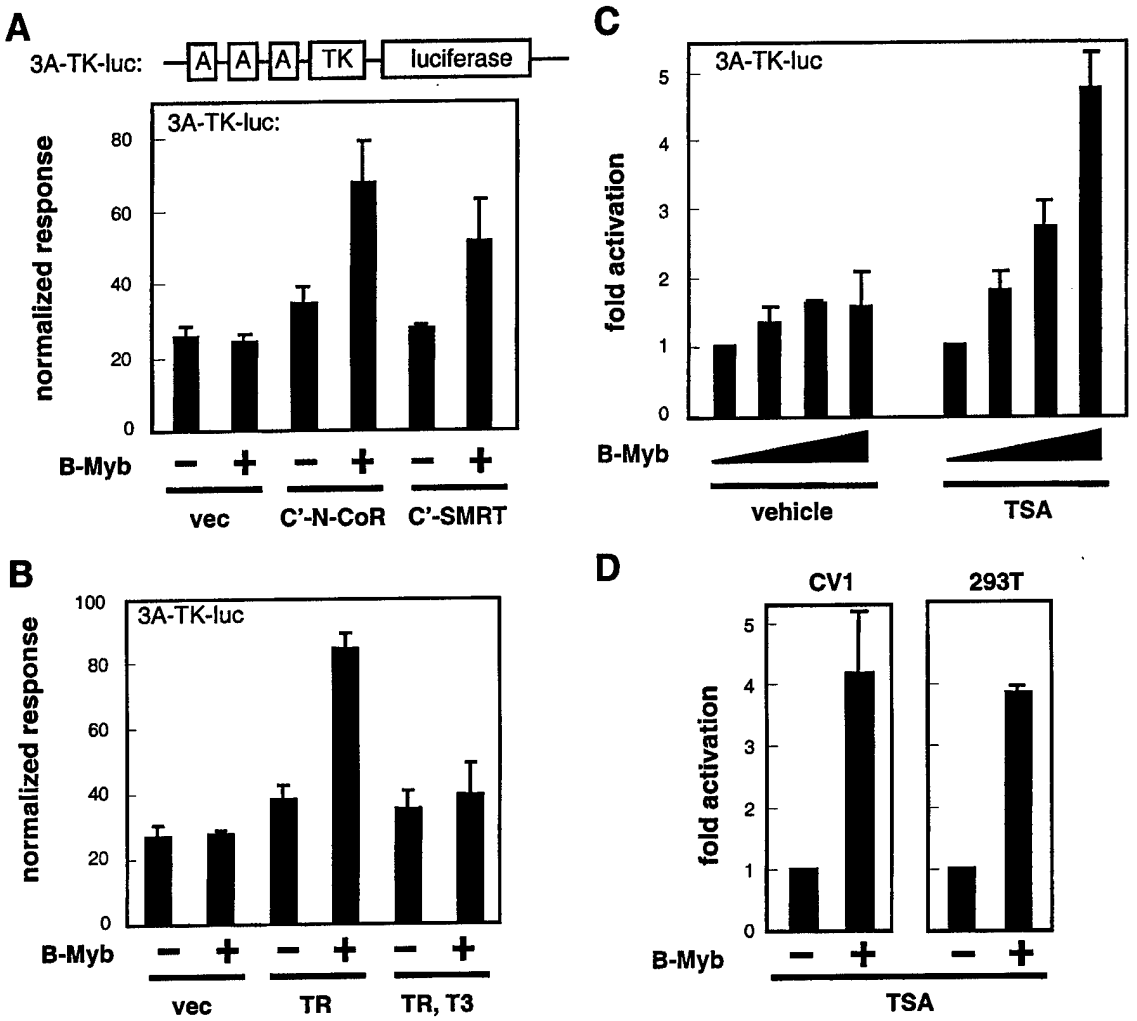

FIG. 3. Inhibition of the repression function of N-CoR and SMRT releases B-Myb transactivation activity. (A) CV1 cells were transfected with 3A-luc reporter, cytomegalovirus β-galactosidase (CMV-β-Gal) internal control vector, and the plasmids pcDNA3–B-Myb, pcDNA3–C'N-CoR, and pCMX–C'SMRT as indicated. Empty pcDNA3 was added to assays to ensure that each assay contained equal amounts of CMV promoters. The result is a representative of three independent assays. (B) CV1 cells were transfected with 3A-TK-luc reporter, CMV–β-Gal internal control vector, and pcDNA3-TRβ. T3, 100 nM. (C) HepG2 cells were transfected with various amounts of pcDNA3–B-Myb. Empty pcDNA3 was added to assays to ensure that each assay contained equal amounts of CMV promoters. TSA, 20 nM. (D) CV1 and 293T cells were transfected with pcDNA3–B-Myb. TSA, 20 nM. Results are representative of three independent assays.

HDAC inhibitors such as TSA should enhance B-Myb transcriptional activity. Indeed, B-Myb displayed significant transcriptional activity in HepG2 cells treated with TSA (Fig. 3C). A similar enhancement of B-Myb transcriptional activity by TSA was also observed in both CV-1 and 293T cells (Fig. 3D). Cumulatively, these data indicate that B-Myb is repressed by a ubiquitously expressed, TSA-sensitive factor(s). Such characteristics are consistent with those of N-CoR and SMRT, further supporting our hypothesis that N-CoR and SMRT are physiological regulators of B-Myb transcriptional activity.

N-CoR and SMRT act through the previously defined negative regulatory domain of B-Myb. It has been shown by others that truncation of the C terminus of B-Myb releases the constitutively repressed B-Myb transactivation activity (19, 36). Consistent with this, using transiently transfected HepG2 cells, we observed that a B-Myb mutant (B-Myb$_{1-561}$) that is truncated at the C terminus displays markedly stronger transactivation activity than the full-length B-Myb (Fig. 4A). We next investigated whether the corepressors SMRT and N-CoR can interact with the B-Myb$_{1-561}$ mutant. Using a mammalian two-hybrid assay, we found that VP16–B-Myb$_{1-704}$ interacts in a robust manner with Gal4–C'N-CoR and Gal4–C'SMRT while VP16–B-Myb$_{1-561}$ displays essentially no corepressor binding activity (Fig. 4B). We confirmed that the VP16–B-Myb$_{1-561}$ fusion protein was properly expressed by demonstrating that it was able to activate transcription of the Myb-responsive 3A-TK-luc reporter (Fig. 6C). Thus, a correlation was established between enhancement of B-Myb$_{1-561}$ transcriptional activity and loss of N-CoR and SMRT binding activity. This correlation was further demonstrated by the observation that unlike that of full-length B-Myb (Fig. 2), the transcriptional activity of B-Myb$_{1-561}$ cannot be enhanced by C'N-CoR, C'SMRT, unliganded TRβ, or TSA (Fig. 4C and D).

Clearly, the repressor activity of B-Myb requires the C ter-

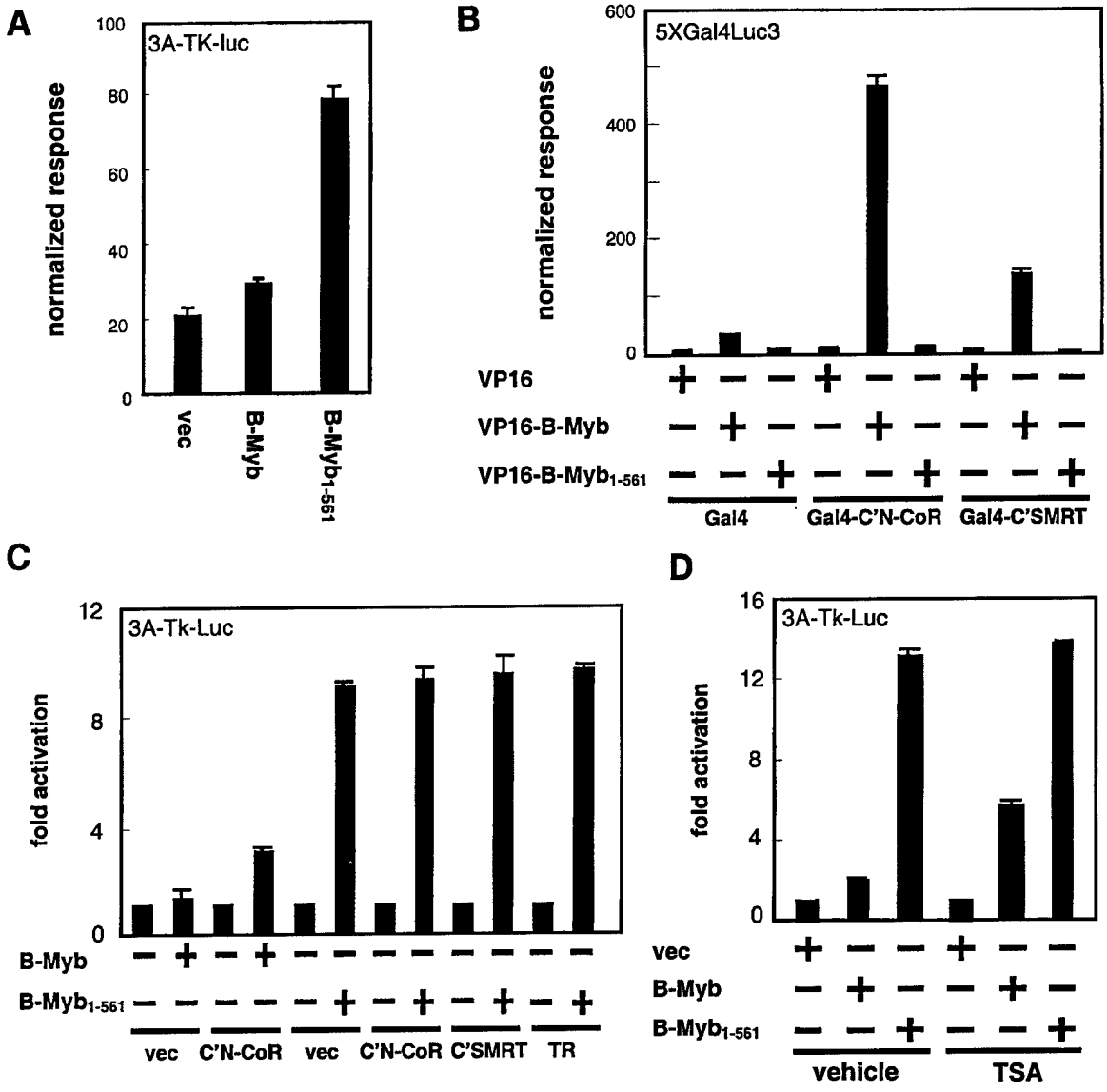

FIG. 4. C-terminal truncation of B-Myb loses N-CoR binding activity. (A) HepG2 cells were transfected with 3A-TK-luc, cytomegalovirus β-galactosidase (CMV–β-Gal), and the plasmids as indicated. Each assay contains equal amounts of CMV promoters. (B) HepG2 cells were transfected with 5XGal4Luc3, CMV–β-Gal, and the plasmids as indicated. The results for the mammalian two-hybrid assays are representative of three independent experiments. (C) HepG2 cells were transfected with 3A-TK-luc, CMV–β-Gal, and the plasmids as indicated. (D) HepG2 cells were transfected with 5XGal4Luc3, CMV–β-Gal, and the plasmids as indicated. TSA (20 nM; vehicle, ethanol) was added 24 h after transfection, and cells were grown for additional 24 h.

minus of the protein, which prompted us to test whether this domain is sufficient for N-CoR interaction. To address this issue, we constructed a series of B-Myb mutants and measured their abilities to interact with N-CoR using a mammalian two-hybrid assay (Fig. 5). The result of this analysis suggests that the minimal requirement for N-CoR binding is B-Myb$_{197-704}$, which includes the transactivation domain (TA), the conserved region (CR), and the negative regulatory domain (NRD). Within B-Myb$_{197-704}$, neither TA, CR, nor NRD showed significant N-CoR binding activity. These data indicate that although truncation of the NRD abolishes N-CoR binding, NRD itself does not comprise the corepressor binding domain. Instead, a much larger region of B-Myb (aa 197 to 704) is necessary to maintain the interaction between N-CoR and B-Myb.

C-Myb does not associate with N-CoR. In addition to B-Myb, the *myb* family of transcription factors has two other members, A-Myb and c-Myb (26, 29, 31). However, the region whose high degree of amino acid homology has been used to group A-, B-, and c-Myb in the same family (the DNA binding domain) does not overlap with the carboxyl-terminal N-CoR interacting domain mapped in B-Myb. In addition, unlike B-Myb, c-Myb is a constitutive transcriptional regulator and thus may not be subject to regulation by a corepressor (26, 29, 31). Regardless, we felt that it was important to determine if N-CoR and SMRT were able to interact with other *myb* family members. Specifically, we evaluated potential interactions between N-CoR and c-Myb. Using the 3A-TK-luc reporter, we were able to show that in HepG2 cells c-Myb displays signifi-

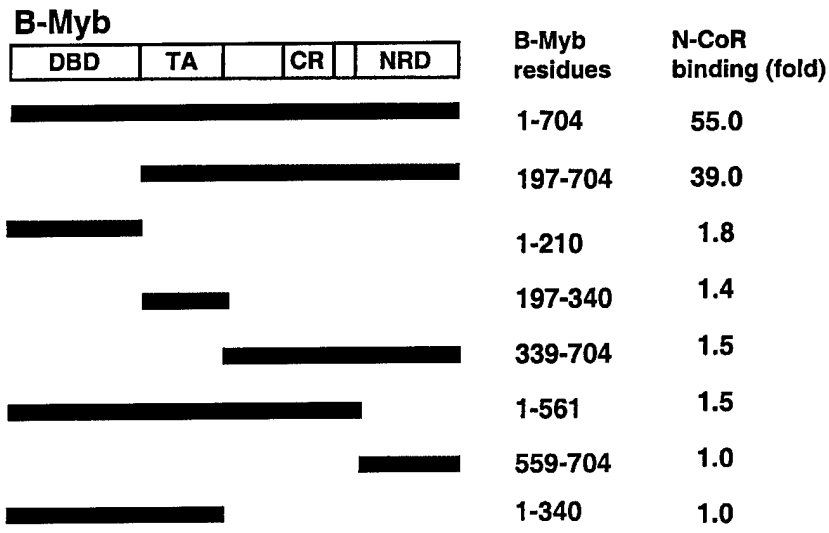

FIG. 5. Several B-Myb domains are required for B-Myb/N-CoR interaction. The domain structure of B-Myb was adapted from findings by Saville and Watson (31). B-Myb DNA binding domain (DBD), transactivation domain (TA), conserved region (CR), and negative regulatory domain (NRD) encompass residues 1 to 210, 197 to 340, 470 to 550, and 561 to 704, respectively. Individual domains, combinations of domains, and complementary regions of such domain(s) were generated as VP16 fusion. Their N-CoR binding activities were measured by their ability to associate with Gal4–C'N-CoR in the mammalian two-hybrid assay in CV1 cells. N-CoR binding (fold) was the fold activation in the two-hybrid assay.

cant transcriptional activity whereas B-Myb activity is completely repressed under the same conditions (Fig. 6A). We further demonstrated by a two-hybrid assay that VP16–c-Myb does not interact with Gal4–C'N-CoR while VP16–B-Myb does (Fig. 6B). Our control experiments (Fig. 6C) indicated that both VP16-B–Myb and VP16–c-Myb are capable of activating the Myb-responsive 3A-TK-luc reporter, suggesting that the VP16–c-Myb fusion protein was properly expressed. Therefore, our data suggest that c-Myb does not possess intrinsic N-CoR binding activity.

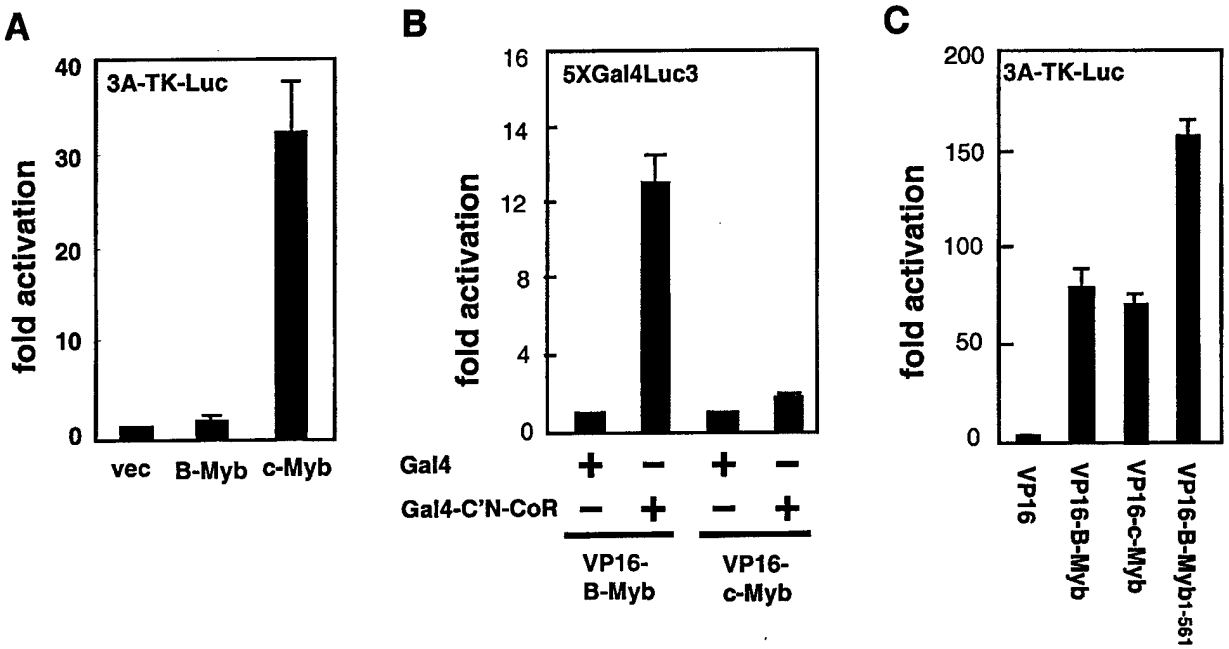

FIG. 6. C-Myb does not associate with N-CoR. (A) HepG2 cells were transfected with 3A-TK-luc, cytomegalovirus β-galactosidase (CMV–β-Gal), and the plasmids as indicated. The plasmid for B-Myb is pcDNA3–B-Myb, and its control vector is pcDNA3. The plasmid for c-Myb is pRMb3SV/c-Myb, and its control vector is pR3SV. Each assay contains equal amounts of CMV promoters. (B) HepG2 cells were transfected with 5XGal4Luc3, CMV–β-Gal, and the plasmids as indicated. The results for the mammalian two-hybrid assays are representative of three independent experiments. (C) HepG2 cells were transfected with 3A-TK-luc, CMV–β-Gal, and the plasmids as indicated.

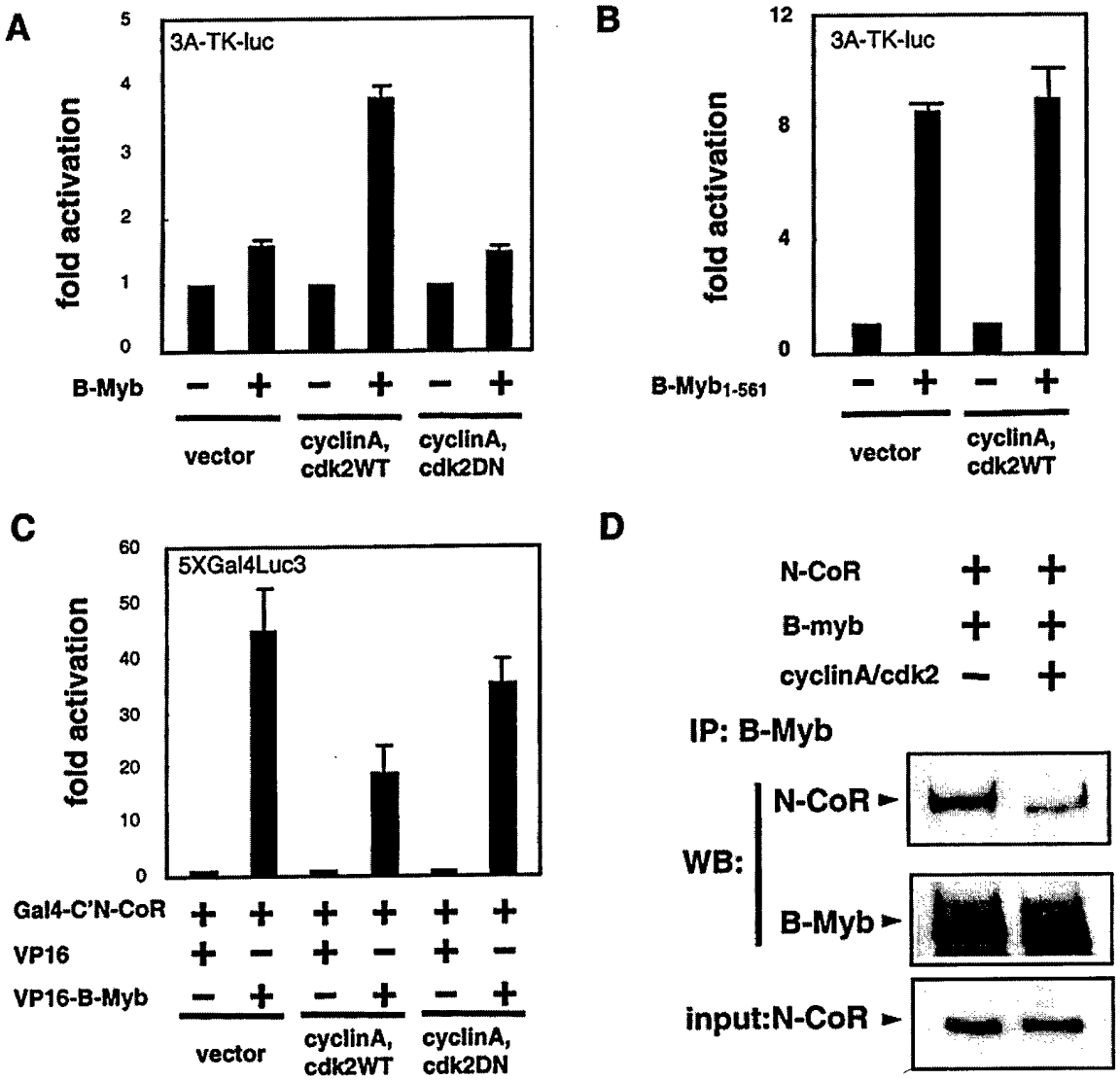

FIG. 7. Cyclin A/cdk2 destabilizes N-CoR–B-Myb interaction. (A) Cyclin A/cdk2 releases B-Myb transactivation activity. HepG2 cells were transfected with 3A-TK-luc, cytomegalovirus β-galactosidase (CMV–β-Gal), pcDNA3-B-Myb, and plasmids as indicated. Results shown are representative of three independent experiments. (B) Effects of cyclin A/cdk2 on the transactivation activity of B-Myb$_{1-561}$. HepG2 cells were transfected with 3A-TK-luc, CMV–β-Gal, pcDNA3–B-Myb, and plasmids as indicated. (C) CV1 cells were transfected with 5XGal4Luc3, CMV–β-Gal, and plasmids as indicated. Results for the mammalian two-hybrid assay are representative of three independent assays. (D) 293T cells were transfected with the plasmids indicated and incubated at 37°C for 40 h. The whole-cell extract was generated for immunoprecipitation with rabbit anti-B-Myb antibody. Mouse anti-myc was used in Western blotting to detect myc–N-CoR$_{759-2453}$. Rabbit anti-B-Myb was used to confirm the precipitated B-Myb.

Cdk2/cyclin A-mediated phosphorylation blocks the ability of B-Myb to interact with corepressors. It has been shown previously that phosphorylation of B-Myb by cdk2/cyclin A enhances its transcriptional activity in U-2OS, SAOS-2, and QT6 cells (1, 19, 28, 37). Consistent with these observations, we found that in HepG2 cells expression of cdk2/cyclin A markedly enhances B-Myb's ability to transactivate the 3A-TK-luc reporter (Fig. 7A). A dominant negative form of cdk2, cdk2DN, that lacks kinase activity has been developed (34). When this protein was expressed with cyclin A in HepG2 cells, it had no effect on B-Myb transcriptional activity (Fig. 7A). The transcriptional activity of B-Myb$_{1-561}$, unlike that of B-Myb, was not affected by cyclinA/cdk2 (Fig. 7B). In fact, the insensitivity of B-Myb upon cyclin A treatment has also been observed by others (6). Since we have shown that B-Myb, but not B-Myb$_{1-561}$, interacts with corepressors N-CoR and SMRT, we hypothesized that cdk2/cyclin A-mediated phosphorylation could affect the interaction between B-Myb and the negative regulatory factors N-CoR and SMRT.

Using a mammalian two-hybrid assay, we found that the interaction between B-Myb and N-CoR is reduced in the presence of overexpressed cdk2/cyclin A (Fig. 7C). No significant reduction in the interaction between B-Myb and N-CoR was observed when the two-hybrid assay was performed in the

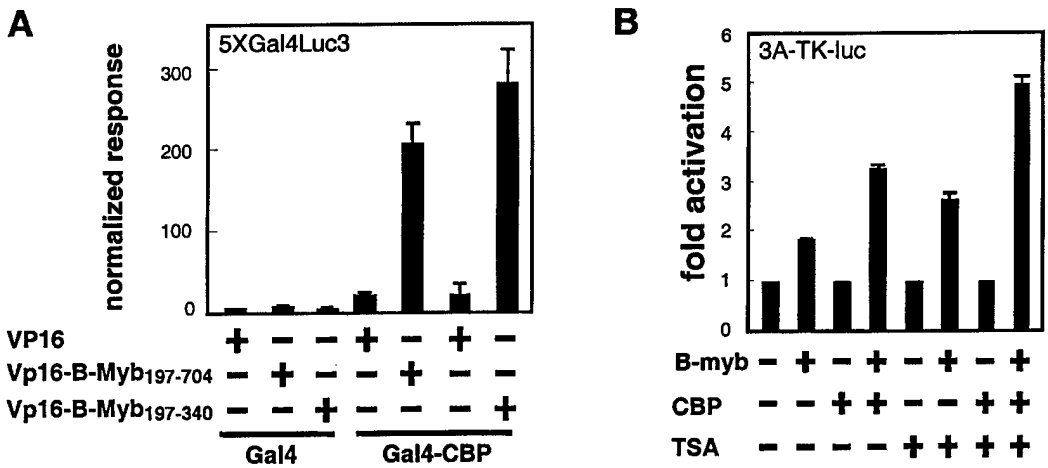

FIG. 8. CBP interacts with B-Myb and potentiates B-Myb transcriptional activity. (A) CBP interacts with B-Myb$_{197-704}$ and B-Myb$_{197-340}$ in a mammalian two-hybrid assay. CV1 cells were transfected with 5XGal4Luc3, cytomegalovirus β-galactosidase (CMV–β-Gal), and the plasmids as indicated. (B) Effects of CBP and TSA on a B-Myb transcription assay. 293T cells were transfected with 3A-TK-luc, CMV–β-Gal, and plasmids as indicated. TSA concentration, 20 nM. Results are representative of three independent assays.

presence of overexpressed cdk2DN. The role of cdk2/cyclin A as a modulator of B-Myb–corepressor interactions was confirmed by using a co-IP assay (Fig. 7D). Specifically, we measured the amount of myc-tagged N-CoR$_{759-2453}$ that was associated with the B-Myb complex in extracts of transfected cells in the presence or absence of overexpressed cdk2/cyclin A. We found that coexpression of cdk2/cyclin A correlates with significantly reduced ability of B-Myb to associate with N-CoR under conditions where N-CoR and B-Myb expression levels were not affected (Fig. 7D). It appears, therefore, that cdk2/cyclin A is a key regulator of the transcriptional activity of B-Myb and that it functions by regulating the ability of the transcription factor to interact with the corepressors N-CoR and SMRT.

CBP interacts with and potentiates B-Myb transcriptional activity. Thus far, our studies have indicated that inhibition of the activity of corepressors N-CoR and SMRT is a key step in the activation of B-Myb. However, the identities of the positive acting factors which interact with the derepressed B-Myb proteins and permit it to activate transcription have not been well characterized. Recently, it has been shown that CBP, a general coactivator protein, can interact with B-Myb (6). We have been able to confirm this finding in our model systems using a co-IP assay to demonstrate that CBP is capable of associating with B-Myb (not shown). These interactions were confirmed in a two-hybrid assay which indicated that B-Myb, specifically a domain spanning residues 197 to 340, was able to interact with CBP (Fig. 8A). This particular region of B-Myb (aa 197 to 340) has previously been shown to constitute the TA of this coactivator. In light of these findings, we considered that it might be possible to increase the dynamic range of B-Myb transactivation by inhibiting the corepressor function of N-CoR and SMRT, thus facilitating the coactivator function of CBP. Consequently, we investigated whether CBP and N-CoR (or SMRT) cooperate in the regulation of B-Myb transactivation activity. The results of our transcription assay suggested that coexpression of CBP stimulates B-Myb transactivation activity (Fig. 8B). When the HDAC inhibitor TSA is added, the activity of B-Myb is enhanced, and further potentiation of B-Myb activity is achieved when both TSA and CBP are present. It appears, therefore, that the activity of B-Myb in target cells may be subject to (i) positive regulation by coactivators such as CBP and (ii) negative regulation by corepressors such as N-CoR (or SMRT).

DISCUSSION

In this study, we have used several experimental approaches to demonstrate that N-CoR and/or SMRT directly interacts with B-Myb and regulates its transcriptional activity. Under most circumstances, B-Myb is maintained in an inhibited state in cells. We were able to relieve this repression and enable B-Myb transcriptional activity by inhibiting the activity of the corepressors using either dominant negative corepressor mutants or pharmacological inhibitors. B-Myb transcriptional activity was also manifest when mutants were introduced into the protein that blocks B-Myb's ability to interact with N-CoR and SMRT. It has been proposed by others that direct phosphorylation of B-Myb by cdk2/cyclin A is the physiologically relevant signaling event that leads to its conversion from an inactive to a transcriptionally active state. Our findings support this hypothesis and demonstrate that phosphorylation decreases the ability of B-Myb to interact with N-CoR. Considering the fact that B-Myb, N-CoR, and SMRT are widely expressed in almost all cells (9, 14, 31) and our observation that endogenous B-Myb and N-CoR can interact, it is likely that the corepressors can function as physiological regulators of B-Myb transcriptional activity.

Based on our findings and those of others, we propose a simple model to explain how B-Myb transcriptional activity is regulated by the corepressors N-CoR and SMRT and by the coactivator CBP (Fig. 9). Specifically, we suggest that under most circumstances B-Myb activity is suppressed as a consequence of its ability to interact with N-CoR or SMRT. However, during the S phase of the cell cycle two distinct events can occur. First, there is an increase in B-Myb expression that may

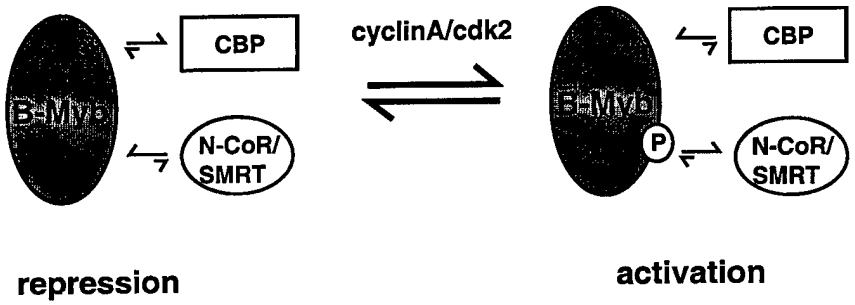

FIG. 9. Schematic model illustrating how N-CoR and SMRT cooperate with CBP in regulating B-Myb transcriptional activity.

enable a titration of the available corepressors and decrease the threshold for activation. Secondly, at the S phase of the cell cycle the cdk2/cyclin A pair can phosphorylate B-Myb, an event that leads to the disruption of the interaction between B-Myb and the corepressors. When the negative influence of the corepressor is removed, CBP is able to interact with the transactivation domain of B-Myb and its positive transcriptional activity is manifest. This model would suggest that alterations in the integrity, expression level, or activity of N-CoR or SMRT would have a profound effect on B-Myb transcriptional activity. We believe that these studies provide compelling evidence that N-CoR and/or SMRT is a physiologically relevant negative regulator of B-Myb transcriptional activity, an association that suggests that these corepressors may be involved in cell cycle regulation.

Studies from others have revealed that there are multiple sites in B-Myb that can be phosphorylated by cdk2/cyclin A (5, 17, 32). Many of these sites are likely to be involved in regulating the interaction of B-Myb with N-CoR and SMRT since mutation of each leads to a progressive increase in the transcriptional activity of this coactivator. A mutation of up to four of the major cdk2/cyclin A phosphorylation sites reduces, but does not totally inhibit, cyclin/cdk2's ability to enhance the transcriptional activity of B-Myb (32). This indicates that there may be additional sites on B-Myb (or in other proteins interacting with B-Myb) where phosphorylation by cdk2/cyclin A is involved in regulating the interaction of this transcription factor with N-CoR. In line with this apparent complexity, we have shown that the N-CoR binding domain on B-Myb is large, comprising most of the carboxyl half of the protein. Although all indications are that B-Myb is the primary target of cdk2/cyclin A phosphorylation in this B-Myb–N-CoR regulatory system, we cannot rule out the possibility that N-CoR may also be subject to cdk2/cyclin A-mediated phosphorylation.

In this study we have found that the transcriptional coactivator CBP interacts directly with the previously defined transactivation domain within B-Myb. We do not know whether the coactivator and the corepressor bind to B-Myb simultaneously or whether displacement of the coactivator is required for subsequent coactivator recruitment. Until recently, the sequential model was favored. However, there is an increasing amount of evidence that suggests that transcriptional repression and activation may be more closely regulated than was previously thought. This position is supported by the recent demonstration that the nuclear receptor cofactor SHARP can interact with both the coactivator SRA and the SMRT/HDAC corepressor complex (33). In another study, p300 (a coactivator) and Groucho (a corepressor) were demonstrated to bind separate domains of the transcription factor NK-4 (10). Of more direct relevance to our studies, however, was the demonstration that the homeobox protein heterodimer Hox-pbx, N-CoR, and CBP exist in a single complex and that PKA-mediated phosphorylation of Hox-pbx by PKA permits this transcription factor to activate target gene transcription (3, 30). Interestingly, PKA-mediated phosphorylation of Hox-pbx enhances its ability to interact with CBP. Whether phosphorylation has any effect on the interaction of corepressors with this transcription factor pair is not known. However, it appears that the regulation of B-Myb transcriptional activity occurs in a manner that is similar to that of other well-characterized transcription factors. Interestingly, we found in a previous study that addition of 8-Br-cAMP, a PKA activator, to cells was sufficient to abolish the interaction between the N-CoR and the human progesterone receptor (35). Thus, phosphorylation may be a general way of displacing N-CoR and SMRT from transcription factors.

In addition to B-Myb, N-CoR and SMRT have been shown to interact with numerous other transcription factors (3, 4, 13, 33). Thus, although originally classified as regulators of the transcriptional activity of nuclear receptors, they are clearly involved in a more diverse array of cellular processes. N-CoR and SMRT are not abundant proteins, and therefore it is likely that alterations in the expression levels or activities of these corepressors would impact several different processes. With respect to the corepressors themselves, it has been shown that genetic disruption of the corepressor N-CoR in mice gives a complicated embryonic lethal phenotype (16). This suggests that N-CoR and SMRT are not able to substitute for each other in all circumstances. In breast tumors that are resistant to the antiestrogen tamoxifen, a previous study has demonstrated that the corepressors are significantly down-regulated (20). Whereas these studies conclude that corepressor down-regulation permits tamoxifen to manifest agonist activity and that this explains tumor progression, it is equally likely that derepression of B-Myb or other transcription factors may also be involved. Given that N-CoR and SMRT interact with different transcription factors in cells, it is possible that a pathological overexpression of any one of these partners could titrate out the available corepressors and enhance the activity of multiple, functionally unrelated, transcription factors. In support of this hypothesis, we demonstrated that overexpression of apo-TR leads to an enhancement of B-Myb transcriptional activity. A

mechanism such as this may help to understand the puzzling observation that overexpression of B-Myb can activate the HSP70 promoter despite the fact that a B-Myb binding site in this promoter has not yet been identified (12).

ACKNOWLEDGMENTS

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APPENDIX 2

Direct Interactions between Corepressors and Coactivators Permit the Integration of Nuclear Receptor-Mediated Repression and Activation

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The unliganded thyroid hormone receptor β (TRβ) represses the basal transcriptional activity of target genes, in part through interactions with ~~core-pressor protein~~ N corepressor (N-CoR). In this study we have identified a rather unexpected interaction between N-CoR and the coactivator ACTR. We have demonstrated *in vitro* and in intact cells that N-CoR directly associates with ACTR and that the interaction surfaces on N-CoR and ACTR are distinct from those required for TR binding. The significance of this finding was demonstrated by showing that N-CoR facilitates an interaction between ~~apolipoprotein~~-TRβ and ACTR. One possible consequence of the formation of the trimeric complex of N-CoR/ACTR/~~apolipoprotein~~-TR is that N-CoR may raise the local concentration of ACTR at target gene promoters. In support of this hypothesis it was demonstrated that the presence of N-CoR can enhance TRβ-mediated transcriptional activation. It is proposed, therefore, that TRβ-mediated activation and repression are integrally linked in a manner that is not predicted by the current models of nuclear receptor action. (*Molecular Endocrinology* 16: 0000–0000, 2002)

THE THYROID HORMONE receptor β (TRβ) is a ligand-inducible transcription factor involved in the regulation of morphogenesis and metabolism (1, 2). The current models of thyroid hormone action suggest that in the absence of ligand the ~~apolipoprotein~~ ~~(apo)~~ receptor binds to specific thyroid hormone response elements (TRE) located within target gene promoters (3–5). In this DNA-bound state, apo-TRβ is capable of nucleating the assembly of a histone deacetylase complex, facilitating local condensation of chromatin and subsequent transcriptional silencing (6–9). Upon ligand binding, the receptor undergoes a conformational change that relieves this repressing activity by displacing the histone deacetylase complex and recruiting a complex that possesses histone acetyltransferase activity (10–12). In this manner the repressive effects of chromatin are overcome, and transcription of the target gene ensues.

Although the components of the complexes involved in transcriptional activation and repression have been identified, and their role in TR action has been defined, little is known about the processes that lead to the exchange of complexes. It is unclear, for instance, whether the proteins involved in transcriptional activation or repression are present in different complexes within the cell or are present in a single complex whose bio-character is influenced by the state of activation of TR. The existing cofactor exchange models, which suggest that biochemically distinct activation and repression complexes exist within cells, imply that ligand-activated TR is presented constantly, with the problem of having to find appropriate cofactors before it can activate transcription. Consequently, the kinetics of target gene activation would be very sensitive to the cellular concentrations of individual components of the activation complex. If, on the other hand, the proteins required for activation and repression are present in the same complex and the role of ligand is merely to reorientate the complex, a more rapid transition to transcriptional activation could occur. Resolving this issue has important implications with respect to TR pharmacology and may help elucidate the roles of agonists and antagonists in modulating nuclear receptor action in general.

RESULTS

N-CoR Interacts Directly with ACTR

To define the biochemical processes that enable TR to function as both a transcriptional activator and a repressor, we wished to study the protein-protein interaction surfaces on the corepressor protein N-CoR which are important for its ability to modulate TR function. Using the repressor domain 3 (RD3) and the receptor interaction domain 1 (ID1) of N-CoR as targets, we performed phage display analysis to identify N-CoR-interacting peptides. The initial screen lead to the identification of N-CoR-interacting peptides, a

Abbreviations: aa, Amino acid; ~~apo, apolipoprotein~~; CBP, CREB ~~corticotropin~~-binding protein; ChIP, chromatin immunoprecipitation; CMV, cytomegalovirus; D1, type 1 iodothyronine deiodinase; ER, estrogen receptor; β-gal, β-galactosidase; GST, glutathione-*S*-transferase; ID1, interaction domain 1; RAR, retinoic acid receptor; RD3, repressor domain 3; ST3, stromelysin-3; TRβ, thyroid hormone receptor β; TRE, thyroid hormone response element; N-CoR, nuclear receptor corepressor; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; GRIP-1, glucocorticoid receptor interacting protein-1; SRC1, steroid hormone receptor coactivator 1

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subset of which surprisingly had similarity to the nuclear receptor coactivator, ACTR (p/CIP/AIB1/RAC3/TRAM-1/SRC-3) (11, 13–17). Although the peptides (not shown) contained relatively weak N-CoR-binding activity, their homology to ACTR intrigued us, as N-CoR and ACTR are both TR cofactors, and they coexist in the same cellular compartment. This prompted us to examine a potential interaction between N-CoR and ACTR using glutathione-*S*-transferase (GST) pull-down experiments. As shown in Fig. 1B, RD3, ID1 alone and a larger region that contains ID1, termed C'N-CoR [amino acids (aa) 1944–2453] all interact with full-length *in vitro* translated ACTR.

F1

Direct interactions between N-CoR and ACTR were also shown to occur in intact cells (Fig. 1C). In transfected 293T cells, we were able to demonstrate that full-length ACTR and N-CoR could be coimmunoprecipitated, using an antibody against ACTR to immunoprecipitate and a Myc antibody to detect the Myc-tagged N-CoR by immunoblot (Fig. 1C, lanes 1 and 2). No signal was detected by the anti-Myc antibody in cells expressing ACTR alone (Fig. 1C, lanes 3 and 4). The interaction between N-CoR and ACTR was also demonstrated in a reciprocal manner in which a Myc-tagged N-CoR fragment (N-CoR$_{759-2453}$) was immunoprecipitated with an anti-Myc antibody, while ACTR was detected in the immunoblots using an anti-ACTR antibody (Fig. 1D). In addition, a significant interaction between full-length versions of ACTR and N-CoR (Gal4-ACTR and VP16-N-CoR) was observed in the mammalian two-hybrid assay (Fig. 2A), further supporting the hypothesis that N-CoR and ACTR interact directly in cells.

F2

Based on the results ~~obtained to date~~ presented thus far, it appears that a surface encompassing at least the RD3 and ID1 domains mediates the interaction between N-CoR and ACTR. Other individual N-CoR domains were not examined for their ACTR-binding ability; nevertheless, we concluded that full-length N-CoR is capable of interacting with ACTR based on results of the ~~coimmunoprecipitate~~ coimmunoprecipitation, two-hybrid, and GST pull-down assays (Figs. 1, B and C, 2A, and 3A). The results also suggest that N-CoR interacts with ACTR through surfaces distinct from those that mediate N-CoR's interaction with TR. We next evaluated the surfaces on ACTR that permit its interaction with N-CoR. Although

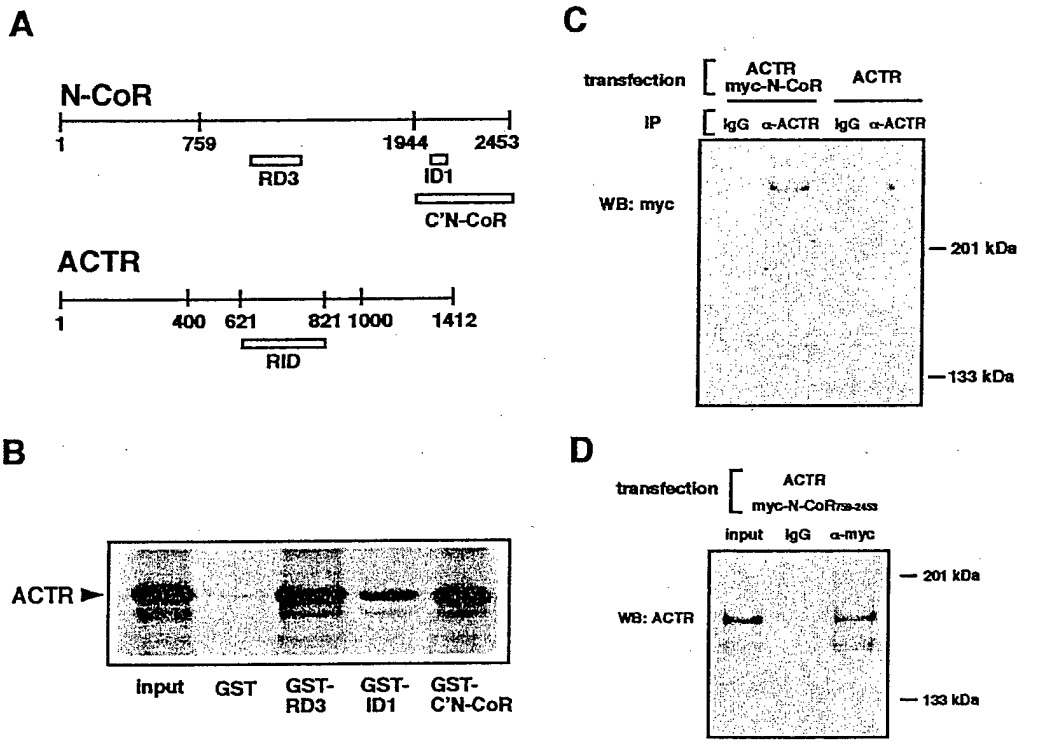

Fig. 1. N-CoR and ACTR Directly Interact *In Vivo* and *In Vitro*

A, Schematic models of domains of N-CoR and ACTR were partially derived from previous reports (7, 11). B, N-CoR domains associate with ACTR in GST pull-down experiments. N-CoR domains RD3 (aa 1017–1461), ID1 (aa 2063–2142), and C'N-CoR (aa 1944–2453) were expressed as GST fusion proteins and isolated by glutathione-conjugated beads. GST alone or GST fusion (5 μg each) was incubated with [^{35}S]ACTR. Input, 10%. The result shown is representative of three independent assays. C, 293T cells (70% confluence) were transfected with plasmids pcDNA3-ACTR and pcDNA3-Myc-N-CoR and were grown for additional 2 d. Whole cell lysates were immunoprecipitated by rabbit anti-ACTR and immunoblotted with mouse anti-myc. D, 293T cells were transfected with plasmids pcDNA3-ACTR and pcDNA3-Myc-N-CoR$_{759-2453}$ and were grown for additional 2 d. Whole cell lysates were immunoprecipitated by rabbit anti-Myc and were immunoblotted with mouse anti-ACTR antibodies. Input, 1%.

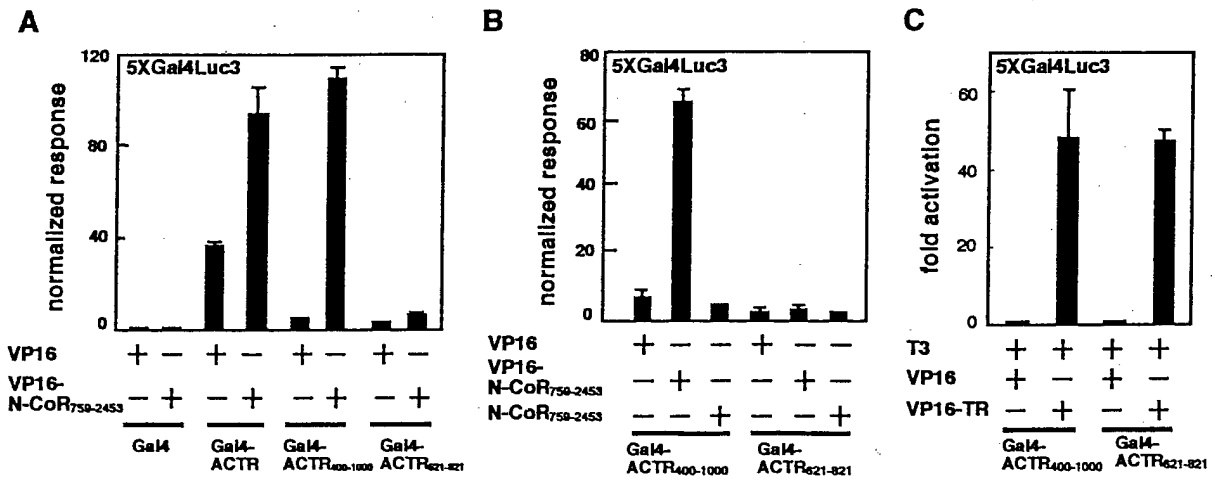

Fig. 2. ACTR Domains Interact with N-CoR

A, ACTR interacts with N-CoR in a mammalian two-hybrid assay. The interaction between the Gal4 and VP16 fusions was measured using a mammalian two-hybrid assay on a 5×Gal4Luc3 reporter gene in CV1 cells. A CMV-β-gal internal control plasmid was used to normalize the luciferase values for transfection efficiency. Protocols for transfection and the luciferase assay are described in *Materials and Methods*. The mean ± SD are shown. B, CV1 cells were transfected with 5×Gal4Luc3 reporter, CMV-β-gal internal control vector, and plasmids as indicated. C, CV1 cells were transfected with 5×Gal4Luc3 reporter, CMV-β-gal internal control vector, and plasmids as indicated. T_3, 10^{-7} M. These results are representative of at least three independent experiments.

full-length ACTR clearly associates with N-CoR in a mammalian two-hybrid assay (Fig. 2A), the high level of basal transcriptional activity exhibited by ACTR when tethered to DNA makes it difficult to evaluate how robust this interaction is. To circumvent this problem in the two-hybrid assays, we used $ACTR_{400-1000}$, a fragment of the coactivator that lacks its ~~cortico~~-tropin-binding protein (CBP) and methyltransferase-binding sites as well as the region responsible for intrinsic (HAT) activity (11, 18). When assayed for its interaction with VP16-N-$CoR_{759-2453}$, the total response of Gal4-$ACTR_{400-1000}$ is equivalent to that of full-length ACTR, yet its fold of induction is much greater due to its lower basal transcriptional activity (Fig. 2A). To rule out the possibility that overexpression of N-CoR has a pan-cellular effect that might indirectly enhance activation, we performed a two-hybrid assay with N-CoR instead of VP16-N-CoR, and no enhancement of ACTR transcriptional activity was observed (Fig. 2B). Thus, the transcriptional activity observed in the two-hybrid assays requires the presence of the VP16 domain on N-CoR and confirms that the activity observed in this assay is indeed an N-CoR-ACTR interaction.

The results of the mammalian two-hybrid assay indicate that $ACTR_{400-1000}$ contains the major N-CoR interaction surface. Also included in $ACTR_{400-1000}$ is the receptor interaction domain or RID (aa 621–821), which is responsible for mediating the coactivator's interaction with transcriptionally active nuclear receptors (11). Consequently, we wanted to determine whether the same domain in ACTR was required for TR and N-CoR interaction. The two-hybrid assays indicated that both Gal4-$ACTR_{621-821}$ and Gal4-$ACTR_{400-1000}$ interact strongly with TRβ (Fig. 2C). However, although $ACTR_{621-821}$ represents the major TR-binding surface, it is not sufficient to allow ACTR to associate with N-CoR (Fig. 2, A and B). We have not ~~been able to create~~ created point mutations that split the surfaces that permit N-CoR-ACTR and N-CoR-TR interactions. However, the studies with deletion mutants indicate clearly that the surfaces within ACTR that interact with N-CoR are distinct from those required for TR binding.

Next, we wanted to determine 1) whether the nuclear corepressor SMRT is able to interact with ACTR, and 2) whether N-CoR and other p160 coactivators could form similar types of complexes. The GST pull-down assay shown in Fig. 3A suggests that SMRT, a corepressor protein homologous to N-CoR, also has the ability to bind ACTR directly. In addition, three members of the p160 family of coactivators, ACTR, SRC-1, and GRIP1, were found to bind N-CoR to various degrees when assayed in mammalian two-hybrid or GST pull-down assays (Fig. 3, B and C). In these assays, ACTR appears to bind N-CoR more avidly than SRC-1. Under the conditions of our assays, GRIP1 displays ~~only marginal~~ little or no N-CoR-interacting activity, ~~which may not be significant~~. These results suggest that the corepressor/coactivator interaction is not limited to N-CoR and ACTR; other cofactors can also participate in this type of interaction. However, the different intrinsic abilities of p160 coactivators to interact with different corepressors (and *vice versa*) could determine, at least in part, the type and number of complexes formed between these two types of proteins.

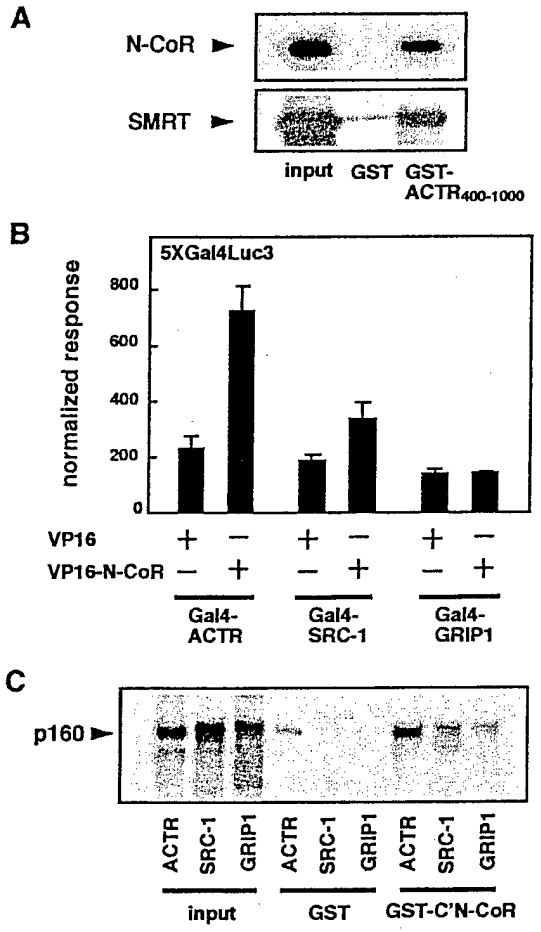

Fig. 3. N-CoR and SMRT Associate with p160 Coactivators

A, ACTR $_{400-1000}$ was expressed as a GST fusion protein and was isolated by glutathione-conjugated beads. [^{35}S]Methionine-labeled corepressors were generated using plasmids pCMX-N-CoR and pCMX-SMRT and were pulled down by 5 μg GST-ACTR$_{400-1000}$. Input, 10%. B, ACTR, SRC-1, and GRIP1 interact with N-CoR in a mammalian two-hybrid assay. CV1 cells were transfected with a 5×Gal4Luc3 reporter, CMV-β-gal internal control vector and plasmids as indicated. The mean ± SD are shown. The results are representative of at least three independent assays. C, [^{35}S]methionine-labeled p160 coactivator proteins were generated using plasmids PCMX-ACTR, pCR3.1-hSRC-1, and pcDNA3-GRIP1. GST or GST-C'N-CoR (5 μg) was incubated with the ^{35}S-labeled p160 proteins. Input, 10%.

N-CoR, ACTR, and Apo-TR Form a Trimeric Complex

Previous studies have shown that N-CoR and TRβ form a complex in the absence of hormone (7). In this study we have shown that both ACTR and TR interact with N-CoR, albeit using different surfaces. Cumulatively, these findings led us to hypothesize that N-CoR, ACTR, and apo-TR may form a trimeric complex in which N-CoR serves as a bridge to link ACTR and unliganded TR. This possibility was first tested *in vitro* using GST pull-down assays. As shown in Fig. 4A, TRβ binds to ACTR in a T_3-dependent manner. However, in the absence of T_3, the addition of N-CoR also resulted in a significant ACTR/TRβ interaction, suggesting that N-CoR can participate in a trimeric complex, possibly by functioning as a bridge between TRβ and ACTR. In the presence of T_3, ACTR and TRβ directly interact, and under the conditions of this assay, the addition of N-CoR has no further potentiating effect.

Additional evidence in support of a trimeric complex was provided using a three-hybrid assay (Fig. 4B), with which we were able to demonstrate that Gal4-ACTR and VP16-TRβ interact in the absence of T_3 only when N-CoR, which contains both the TR and ACTR-binding regions, was coexpressed. We also demonstrated that ACTR's N-CoR-binding ability is required for ACTR to associate with unliganded TR. Specifically, ACTR$_{400-1000}$, but not ACTR$_{621-821}$, was able to interact with unliganded TR in the presence of N-CoR (Fig. 4B). As we have observed previously (Fig. 2B), Gal4-ACTR$_{621-821}$ is properly expressed in cells and displays similar TR-binding activity to ACTR$_{400-1000}$. However, ACTR$_{621-821}$ lacks the ability to associate with N-CoR, which is probably the reason that it cannot participate in the trimeric complex. When interpreting the results of the three-hybrid assay, we also considered the minor possibility that overexpression of the corepressor protein N-CoR might indirectly affect the basal transcription level of Gal4-ACTR. This possibility seems unlikely because N-CoR had no effect in the assay where the VP16-TRβ expression plasmid was replaced by one expressing TR alone (Fig. 4B, last lane). We conclude, therefore, that VP16-tagged TR was physically recruited to Gal4-ACTR by N-CoR.

To further demonstrate that the binding between Gal4-ACTR and apo-VP16-TRβ depends on the bridging function of N-CoR and is not a consequence of some inherent ability of apo-VP16-TRβ to bind Gal4-ACTR, we repeated the experiment using the TRβ AF-2 mutant L454R. It has been shown previously that L454R is unable to interact with the p160 coactivators (19). We also observed that L454R retains N-CoR-binding activity, but does not have the ability to bind ACTR, with or without thyroid hormone (Fig. 4C). In our three-hybrid assay we have found that coexpression of N-CoR allows the TR mutant L454R to interact with ACTR indirectly, and that this interaction is entirely dependent on the presence of N-CoR (Fig. 4D). Nearly identical results were obtained with another TRβ AF2 mutant, E457K (data not shown). These findings strengthen our hypothesis that TRβ and ACTR can interact with each other in an indirect manner in the absence of thyroid hormone by simultaneously binding to N-CoR.

N-CoR and ACTR Coordinate to Regulate TR Action

It is well established that in the absence of thyroid hormone, TR/N-CoR complexes reside on TREs within target gene promoters (1, 2, 7). Our data, which iden-

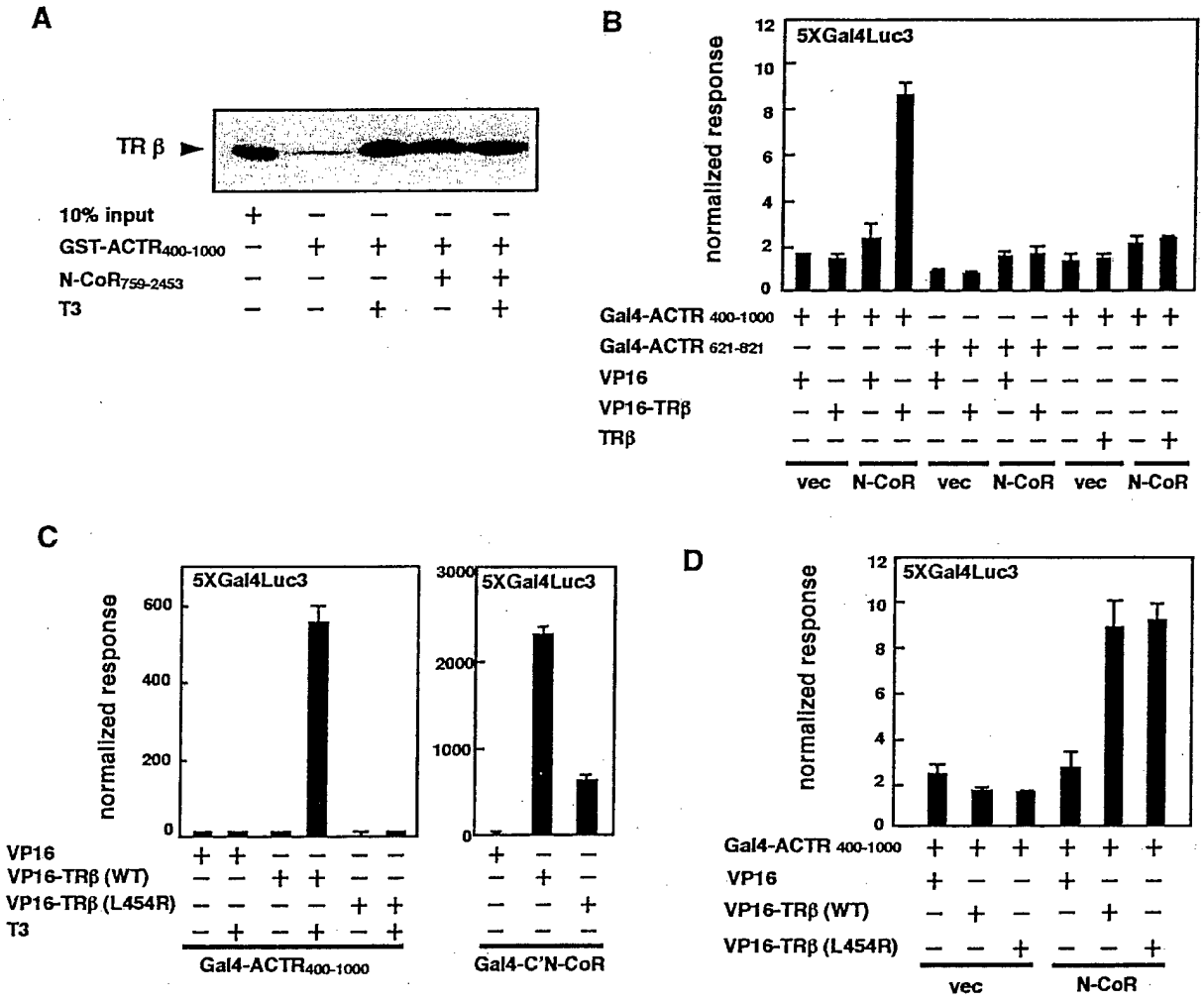

Fig. 4. N-CoR, ACTR, and Apo-TR Form a Trimeric Complex

A, N-CoR promotes an ACTR/TR interaction in a GST pull-down assay. GST-ACTR$_{400-1000}$ was isolated using glutathione-conjugated Sepharose beads and was used to pull down ^{35}S-labeled TRβ. *In vitro* translated nonlabeled N-CoR$_{759-2453}$ or an equal amount of reticulocyte lysate was added to each assay. T_3, 10^{-6} M. B, N-CoR promotes ACTR/TR interaction in the mammalian two-hybrid assay. CV1 cells were transfected with 5×Gal4Luc3, CMV-β-gal, and the plasmids as indicated. pcDNA3-N-CoR or an equivalent molar amount of pcDNA3 vector was added to each assay. The results are representative of three independent assays. C, The TR AF2 mutant (L454R) does not interact with ACTR, but remains able to interact with N-CoR. CV1 cells were transfected with 5×Gal4Luc3, CMV-β-gal, and the plasmids as indicated. Twenty-four hours after transfection, cells were treated with T_3 (10^{-7} M). D, N-CoR promotes ACTR/TR interaction in the mammalian two-hybrid assay. CV1 cells were transfected with 5×Gal4Luc3, CMV-β-gal, and the plasmids as indicated. pcDNA3-N-CoR or an equivalent molar amount of pcDNA3 vector was added to each assay. The results are representative of three independent assays.

tified a trimeric complex of TR/N-CoR/ACTR, implied that ACTR, via association with the TR/N-CoR complex, might also occupy TREs even in the absence of thyroid hormone. To probe this issue further we used chromatin immunoprecipitation (ChIP) assays to study the interaction of TR, N-CoR, and ACTR with the promoter regions of human type 1 iodothyronine deiodinase (D1) and human stromelysin-3 (ST3) genes, both of which contain functional TREs (20, 21). Cross-linked chromatin was immunoprecipitated by specific antibodies and was analyzed using PCR with primers encompassing these endogenous TREs. The results of the ChIP assay suggested that in the absence of thyroid hormone, promoter regions containing TREs in both D1 and ST3 genes can be occupied by ectopically expressed TR, N-CoR, and, more interestingly, ACTR (Fig. 5A).

We subsequently tested the hormone dependency of these factors on TREs. The results indicate that N-CoR occupies the D1 promoter in the absence of T_3, but not when T_3 is added (Fig. 5B). Importantly, ACTR is able to occupy the promoter in the absence of T_3, and the occupancy is enhanced by addition of hormone (Fig. 5C). To confirm the specificity of the ChIP assays, we performed a parallel control assay using primers located approximately 3 kb upstream of the

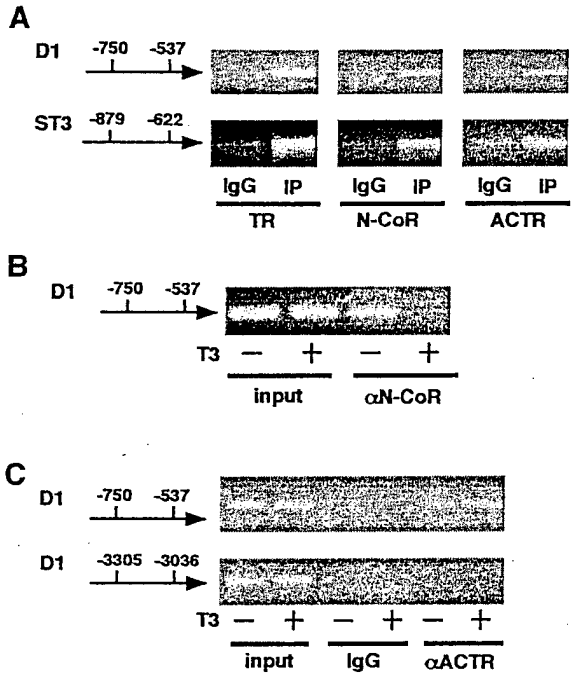

Fig. 5. ACTR, N-CoR, and Apo-TR Occupy Promoters of Thyroid Hormone-Responsive Genes

A, Promoter occupancy by TR and cofactors. Cultured 293T cells were transfected with pcDNA3-TRβ, pcDNA3-ACTR, and pcDNA3-myc-N-CoR and were incubated in medium containing charcoal-stripped serum for 40 h. Soluble chromatin was prepared and immunoprecipitated with control IgG or antibodies against TR, Myc, or ACTR. Extracted DNA from the precipitated complex was subjected to PCR using primers that cover the promoter regions of the D1 and ST3 genes as indicated. B, 293T cells were transfected with pcDNA3-TRβ and were incubated for 40 h. T_3 (10^{-7} M) was applied 2 h before fixation of cells. Soluble chromatin was prepared and immunoprecipitated with anti-N-CoR (C-20). Extracted DNA from the precipitated complex was subject to PCR using primers that cover the TRE regions of the D1 genes. C, The distal region of the D1 gene promoter was examined for the presence of ACTR. Cells and soluble chromatin were prepared as described in B. Rabbit IgG or rabbit anti-ACTR was used for the immunoprecipitation. The results are representatives of at least three independent chromatin preparations and at least three PCR reactions.

TRE, and we were unable to detect any significant binding to the region of the promoter (Fig. 5C). Cumulatively, these results suggest that ACTR together with apo-TR and N-CoR can specifically occupy regions of TREs and that ACTR can reside in a protein complex at target gene promoters before ligand activation.

Our results to date suggest that in the absence of ligand, N-CoR functions not only to suppress TR-mediated gene expression, but also to raise the local concentration of ACTR, possibly to enable activated TRβ to more efficiently activate transcription. To test this hypothesis, we performed transient transfection with a TRE-containing reporter plus plasmids expressing TRβ, ACTR, and N-CoR (Fig. 6A). Transfected cells were grown without hormone to allow formation of the N-CoR/ACTR/TR complex. Subsequently, T_3 was added to the cells, and the TR-mediated transcriptional activity was measured after an additional period of incubation. In this cell system we observed that TRβ was an effective activator of the ~~TRE-containing reporter TRE-PAL~~, and that this activity was enhanced modestly when a submaximal level of ACTR was expressed ectopically. Importantly, however, coexpression of N-CoR and ACTR, but not N-CoR alone, significantly enhanced TRβ-mediated activation. The basal activity of apo-TR in the presence of ACTR and/or N-CoR was not significantly changed over that with TR alone (Fig. 6A). Western blot analysis was used to confirm that overexpression of N-CoR does not affect the expression level of either TRβ or ACTR (Fig. 6B). It appears, therefore, that N-CoR increases the dynamic range of TR transcriptional activity by enhancing its ability to suppress the basal activity of TRE-containing genes in the absence of hormone (7, 9). Yet, at the same time, it can recruit ACTR to the promoter before hormone binding to TR and thus facilitate a rapid and robust response to agonist activation.

Previous studies have shown that upon ligand activation the interaction between N-CoR and TRβ is lost, and a strong interaction between liganded TRβ and a coactivator ensues. Therefore, we sought to determine the effect of ligand-activated TRβ on the interaction between ACTR and N-CoR. As shown in Fig. 6C, addition of ligand-activated TRβ destabilized the interaction between Gal4-ACTR and VP16-N-$CoR_{759-2453}$, whereas apo-TRβ had no effect on the corepressor/coactivator interaction (lanes 6 and 4, respectively). We propose that formation of the liganded TRβ/ACTR complex destabilizes N-CoR/ACTR interaction. Accordingly, TR mutants that cannot associate with ACTR in the presence of ligand should not be able to interfere with the N-CoR/ACTR interaction. Indeed, Fig. 6C, lane 10, shows that the mutant TR L454R cannot disrupt N-CoR/ACTR interaction upon ligand activation. Overall, our experiments with wild-type and mutant TRβs suggest that N-CoR, ACTR, and apo-TRβ can form a trimeric complex, with N-CoR serving as the essential bridging factor between TR and ACTR (Fig. 4). Furthermore, upon addition of hormone, the strong and direct interaction that occurs between TRβ and ACTR contributes to destabilization of the N-CoR/ACTR interaction and possibly to the ejection of N-CoR from the receptor/coactivator complex.

Based on our findings and those of others we have developed a schematic model to describe how the N-CoR/ACTR/TRβ complex may be involved in the regulation of thyroid hormone action (Fig. 6D). We propose that in the absence of thyroid hormone, TRβ resides on a TRE within the promoter of target genes. The apo-receptor is then capable of recruiting the N-CoR/ACTR complex. Upon binding T_3, TRβ undergoes a conformational change that abolishes the N-CoR/TRβ interaction and favors the association of ACTR with the TRβ coactivator-binding pocket. Thus,

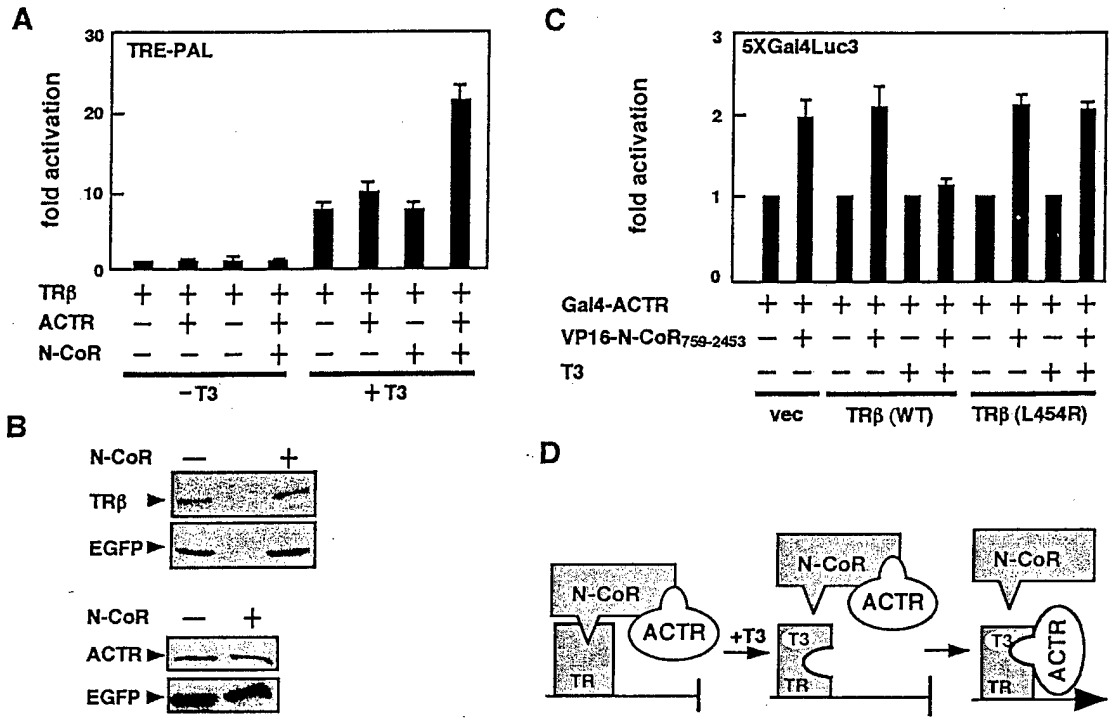

Fig. 6. N-CoR and ACTR Coordinate to Regulate TR Transcriptional Activation

AQ: Q

A, TRE-PAL reporter, CMV-β-gal, pcDNA3-TRβ, pcDNA3-ACTR, and pCMX-N-CoR were cotransfected into CV-1 cells. The control vector pcDNA3 was added to insure that each assay contained equal amounts of CMV promoters. After 30-h incubation, T_3 (10^{-7} M) was added, and cells were incubated for an additional 12 h. Fold activation was calculated using the reference assay points (lane 1) in which apo-TRβ without ACTR or N-CoR was transfected. The results are representative of at least three independent experiments. B, N-CoR does not affect the expression levels of ACTR or TRβ. Expression plasmids for ACTR or TR with or without N-CoR were used in the transient transfection of 293T cells, and 40 μg whole cell extracts were loaded into each lane and probed with rabbit anti-TRβ or rabbit anti-ACTR. Enhanced green fluorescence protein levels serve as internal controls for equal loading. C, CV1 cells were transfected with 5×Gal4Luc3, CMV-β-gal, and the plasmids indicated. VP16 and pcDNA3 vectors were used to insure that each assay contained equivalent amounts of VP16 and CMV promoters. D, A model to explain how N-CoR and ACTR facilitate thyroid hormone action.

formation of the apo-TRβ/N-CoR/ACTR complex facilitates the localization of ACTR close to TR at TREs before ligand activation. This model suggests that the processes of activation and repression are more closely linked than originally anticipated and provides a mechanism to explain the differences in cellular responses to thyroid hormone.

DISCUSSION

The established models of TRβ action hold that in the absence of ligand, the receptor is associated with the corepressor N-CoR (or SMRT) and its associated proteins (6, 7). This complex is capable of associating with specific thyroid hormone response elements within the regulatory regions of target genes, suppressing their basal transcriptional activity. The conformational change within TR that occurs upon ligand binding disrupts this complex, facilitating the interaction of TR with coactivators such as ACTR (11) and enabling the receptor to activate target genes. Our findings generally support this model, with the exception that we have demonstrated that the processes of TR-mediated repression and activation are both functionally and physically linked. Thus, while facilitating TR-mediated repression, the corepressor N-CoR also helps to recruit the coactivator ACTR to the apo-receptor, positioning it to respond to activating ligands.

Using a variety of different approaches, including GST pull-down, mammalian two-hybrid, and immunoprecipitation assays, we have defined a novel, direct interaction between the corepressor N-CoR and coactivator ACTR. Furthermore, we show that this corepressor-coactivator complex is associated with unliganded TR through concurrent binding of TR and ACTR to N-CoR, with N-CoR acting as a bridge between the receptor and coactivator. The prerecruitment of ACTR to the apo-TR by N-CoR appears to facilitate a more robust activation of TR upon ligand treatment. Cumulatively, these experimental findings suggest that TR-mediated activation and repression are functionally and physically linked. It is likely that the amount of coupling between corepressors and

coactivators and its impact on TR activity will depend on the expression levels of various cofactors and these may vary in different cell types. In support of this, we have shown that other p160 coactivators do not interact with N-CoR as well as ACTR does, while the corepressor SMRT can interact with ACTR just as well as N-CoR does. We propose, therefore, that activation and repression are integrally linked but the degree of coupling between the two processes is sensitive to alterations in the cellular expression levels of coactivators or corepressors. Accordingly, our findings may help explain the complex phenotypic differences between siblings with identical TRβ-mutations in Resistance to Thyroid Hormone syndrome (22). It is possible that the variable penetrance of the mutant phenotype may be influenced by the relative expression of ACTR, N-CoR, and other proteins involved in formation of the N-CoR/ACTR interface.

We believe that the paradigm we suggest here, direct coupling of activation and repression, will be found to occur in other systems. In support of this hypothesis, close links between activation and repression have already been implicated in other studies. In one recent study, p300 (a coactivator) and Groucho (a corepressor) were demonstrated to bind separate domains of the transcription factor NK-4, suggesting that the coactivator, corepressor, and transcription factor may be in the same complex (23). Likewise, N-CoR and the coactivator CBP have been shown to bind the homeobox heterodimer pbx-hox simultaneously, while protein kinase A stimulation of CBP has been found to facilitate the switch from transcriptional repression to activation in this system (24, 25). Furthermore, a cofactor protein called SHARP was recently found to interact with both the coactivator SRA and the SMRT/HDAC$_3$ corepressor complex (26). Similar to N-CoR, SHARP is a large protein that has distinct coactivator and corepressor interacting domains. It is therefore plausible that N-CoR and SHARP are similar types of scaffolding proteins, each one interacting with and coordinating multiple coregulators of gene transcription. Such findings are consistent with our hypothesis that corepressors and coactivators coexist in a single regulatory unit.

In this study we have not addressed whether other nuclear receptors, such as retinoic acid receptor (RAR) and estrogen receptor (ER), can participate in similar partnerships with N-CoR and ACTR. RAR uses N-CoR and ACTR as coregulators like TR; therefore, it is possible that RAR integrates transcriptional repression and activation in a way similar to TRβ. Interestingly, N-CoR was recently found to be required for retinoic acid-responsive transcriptional activation of some genes (27). Shang *et al.* (28) have shown that ACTR rapidly associates with ERα upon ligand addition, whereas other coactivators, such as CBP and pCAF, are recruited later. The importance of ACTR and N-CoR in ER action is becoming more evident as several studies have shown that the relative expression levels of these two proteins may play an important role in the pathology of some ER-positive breast tumors. Specifically, a recent study by Anzick *et al.* (29) has shown that the level of ACTR is significantly increased in ER-positive breast cancer cells, while another study has shown that the level of N-CoR is decreased (29). Thus, the net increase in ACTR with no repressor attached in cells might elevate the basal transcriptional activity of certain genes, which could have pathological consequences. Our study with N-CoR, ACTR, and TRβ, along with the findings of others discussed above strongly suggest that corepressors and coactivators can reside together in the same complex and that transcriptional repression and activation are more closely integrated than previously thought.

MATERIALS AND METHODS

Plasmids

The plasmids pCMX-ACTR and pCMX-SMRT were provided by Dr. R. M. Evans (The Salk Institute, La Jolla, CA). Plasmid pCMX-N-CoR was provided by Dr. M. G. Rosenfeld (University of California, San Diego, CA). Plasmids cytomegalovirus (CMV)-TRβ (L454R) and pCMV-TRβ (E457K) were provided by Dr. B. L. West (University of California, San Francisco, CA). Plasmid pcDNA3-GRIP1 was obtained from Dr. C.-Y. Chang (Duke University, ~~Raleigh~~, NC). Gal4-SRC-1 was obtained from Dr. S. A. Onate (University of Pittsburgh, Pittsburgh, PA). Plasmid pcDNA3-5 Myc and pcDNA3-EGFP were obtained from Dr. Maria Huacani-Hamilton (Duke University). VP16-N-CoR$_{759-2453}$ was subcloned using *Xho*I and *Xba*I sites from pCMX-N-CoR to a VP16 vector (~~CLONTECH~~ Laboratories, Inc., Palo Alto, CA). Myc-N-CoR$_{759-2453}$ was subcloned using *Eco*RI and *Not*I sites from VP16-N-CoR$_{759-2453}$ to pcDNA3–5Myc vector. Gal4-ACTR$_{400-1000}$ and Gal4-ACTR$_{621-821}$ were generated by PCR of corresponding regions of ACTR and cloned site into a pM vector (~~CLONTECH~~ Laboratories, Inc.). GST fusion plasmids for N-CoR RD3 (aa 1017–1461), ID1 (aa 2063–2142), and C′N-CoR (aa 1944–2453) were generated by PCR of the corresponding region of N-CoR and cloned into the pGEX-6P-1 vector (Pharmacia Biotech, Piscataway, NJ).

GST Pull-Down Assay

GST fusion proteins were expressed in bacterial strain BL21 and were isolated by glutathione-conjugated Sepharose 4B beads (Pharmacia Biotech). [^{35}S]Methionine-incorporated proteins were generated by TNT kit (Promega Corp., Madison, WI). The bead-coupled GST fusion proteins were incubated with ^{35}S-labeled protein in NETN buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, and 0.5% Nonidet P-40] for 16 h at 4 C. Bound proteins were washed twice with NETN buffer and twice with buffer A [2 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, and 0.5% Nonidet P-40] and were analyzed by SDS-PAGE and autoradiography.

Cell Culture and Transfection

All cultured cells were maintained in the minimum essential medium (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate. Culture dishes were precoated with 0.1% gelatin for 10 min at 25 C. Cells were grown at 37 C in 5% CO_2. Transient transfections were performed using lipofectin reagent (Life Technologies,

Inc.). Protocols for transient transfection and luciferase assays were previously described (30). Briefly, cells were split into 16-mm culture dishes (for immunoprecipitation) or 24-well plates (for luciferase assay) 1 d before the transfection. The lipid-mediated transient transfection was performed with a mixture of lipofectin (Life Technologies, Inc.) and plasmid DNA containing 3 μg DNA for a triplicate luciferase assay in a 24-well plate (Corning, Inc.) or 18 μg DNA for a 60-mm dish (Falcon). Cells were incubated with the lipofectin-DNA mixture for 3–7 h and were then incubated in normal medium for an additional 24–48 h. In the luciferase assays, luciferase readings were normalized using signals of β-galactosidase (β-gal), and the final results are shown as the mean ± SD of triplicate measurements.

Immunoprecipitations and Immunoblots

Cultured cells were washed with PBS and lysed with buffer T containing 20 mM Tris-HCl (pH 7.4), 120 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM Na_3VO_4, and protease inhibitors (Roche Molecular Biochemicals, Indianapolis, IN) for 30 min on ice. The whole cell lysates were clarified by centrifugation and were then precleared for 1 h at 4 C by IgG and protein A agarose (Zymed Laboratories, Inc., San Francisco, CA). Specific antibody was mixed with lysates overnight at 4 C. Protein A beads were added for 2 h and were then washed twice with buffer T and twice with PBS. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to a Hybond-C nitrocellulose membrane (Amersham Pharmacia Biotech, Arlington Heights, IL). The membrane was blocked with a buffer containing 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 5% nonfat dried milk for 1 h. Primary antibody (1–3 μg) was diluted in PBS plus 0.1% Tween 20 and was incubated with the membrane for 2 h at 25 C or overnight at 4 C. Subsequently, the secondary antibodies (1:4000 diluted) were incubated with the membrane for 1 h at 25 C. Anti-Myc mouse monoclonal (9E10), anti-N-CoR goat polyclonal (C-20), and anti-TRβ mouse monoclonal (J51) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-ACTR (anti-AIB1) mouse monoclonal antibody was purchased from BD Transduction Laboratories (Lexington, KY). Anti-ACTR rabbit polyclonal was a gift from Dr. J. Wong (31).

Chromatin Immunoprecipitation

293T cells (90% confluence) were cross-linked, lysed, and immunoprecipitated essentially as previously described (28, 32). Rabbit IgG and rabbit anti-Myc (A14) were obtained from Santa Cruz Biotechnology, Inc. Rabbit anti-TRβ was purchased from Upstate Biotechnology, Inc. Rabbit anti-ACTR was obtained from Dr. J. Wong (31). Precipitates were eluted/reversed by 1% SDS, 0.1 M $NaHCO_3$, and 2 μg/ml herring sperm DNA at 65 C for 6 h. Eluted DNA was isolated by a PCR purification kit (QIAGEN, Chatsworth, CA). PCR was performed with Vent (exo^-) polymerase (New England Biolabs, Inc., Beverly, MA), 5 μl (from a total of 50 μl) eluted DNA, and 30–35 cycles of amplifications. PCR products were resolved in 2.5% agarose/TBE gel and visualized with ethidium bromide. The results shown are representatives of at least three independent chromatin preparations and multiple PCR reactions. Primers for the D1 promoter were: forward, GCTAGAAGCCATGATTGGG; and reverse, TTATCCTGCCTCAACCTCCTG. Primers for the ST3 promoter were: forward, TCTATCCCAAGCTGAAGAACTGGCCAGTCCCTGC; and reverse, CAAGTAGCTGGGACCACAGACGTGCGCCACCATG.

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