Differential Effects of Nuclear Receptor Corepressor (N-CoR) Expression Levels on Retinoic Acid Receptor-Mediated Repression Support the Existence of Dynamically Regulated Corepressor Complexes

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Thyroid hormone and retinoic acid receptors are members of the nuclear receptor superfamily of ligand-dependent transcription factors that stimulate the transcription of target genes in the presence of activating ligands and repress transcription in their absence. Transcriptional repression by the thyroid hormone and retinoic acid receptors has been proposed to be mediated by the nuclear receptor corepressor, N-CoR, or the related factor, SMRT (silencing mediator of retinoic acid and thyroid hormone receptors). Recent studies have suggested that transcriptional repression by N-CoR involves a corepressor complex that also contains mSin3A/B and the histone deacetylase, RPD3. In this manuscript, we demonstrate that transcriptional repression by the retinoic acid receptor can be either positively or negatively regulated by changes in the levels of N-CoR expression, suggesting a relatively strict stoichiometric relationship between N-CoR and other components of the corepressor complex. Consistent with this interpretation, overexpression of several functionally defined domains of N-CoR also relieve repression by nuclear receptors. N-CoR is distributed throughout the nucleus in a nonuniform pattern, and a subpopulation becomes concentrated into several discrete dot structures when highly expressed. RPD3 is also widely distributed throughout the nucleus in a nonuniform pattern. Simultaneous imaging of RPD3 and N-CoR suggest that a subset of each of these proteins colocalize, consistent with the existence of coactivator complexes containing both proteins. In addition, a substantial fraction of both N-CoR and mSin3 A/B appear to be independently distributed. These observations suggest that interactions between RPD3 and Sin3/N-CoR complexes may be dynamically regulated. (Molecular Endocrinology 11: 682–692, 1997)

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INTRODUCTION

Thyroid hormone and retinoic acid receptors are members of the nuclear receptor superfamily of ligand-dependent transcription factors that control diverse aspects of development and homeostasis by regulating the expression of target genes (1, 2). In addition to activation of gene expression in the presence of thyroid hormone (T_3) or retinoic acid, respectively, thyroid hormone and retinoic acid receptors are representa-

tive of a subset of nuclear receptors that repress transcription in the absence of regulatory ligands (3–10). This ligand-independent repression function has been localized to the C-terminal ligand-binding domain (3, 4, 8, 11). Fusion of the ligand-binding domains of the thyroid hormone or retinoic acid receptor to the DNA-binding domain of GAL4 results in chimeric proteins that are capable of repressing transcription from promoters containing multimerized GAL4-binding sites (8). Addition of activating ligands switch the ligand-binding domains of the thyroid hormone and retinoic acid receptors from transcriptional repressors to activators. These observations suggested that unliganded receptors interact with corepressor complexes that are replaced by a set of coactivator complexes that mediate transcriptional activation after the binding of activating ligands.

Biochemical studies of cellular proteins that were capable of interacting with the unliganded thyroid hormone and retinoic acid receptors led to the identification of a 270-kDa protein, termed N-CoR (nuclear receptor corepressor), that exhibited several properties suggesting that it might serve a role as a corepressor (12, 13). N-CoR interacted with the ligand-binding domains of both the thyroid hormone and retinoic acid receptors and was released from DNA-bound receptors by T_3 and retinoic acid, respectively. Interaction of N-CoR with the thyroid hormone and retinoic acid receptors required a region within the N-terminal end of the ligand-binding domain, termed the CoR box (12, 13). Mutations within this region of the thyroid hormone and retinoic acid receptors abolished ligand-independent repression, suggesting that interaction with N-CoR is required for repression function. The isolation of cDNAs encoding N-CoR indicated that the primary transcript can be alternatively spliced to generate several distinct protein products (13, 14). Western blotting experiments indicated that the major protein products migrate at approximately 270 kDa, consistent with the forms initially identified to interact with the retinoic acid and thyroid hormone receptors (12, 13). Functional analysis of N-CoR deletion mutants indicate that it contains two distinct C-terminal domains required for interactions with nuclear receptors (14, 15). Fusion of N-CoR to the DNA- binding domain of GAL4 resulted in a chimeric protein that strongly repressed the transcription of a promoter containing GAL4-binding sites (13). Analysis of subregions of N-CoR linked to the GAL4 DNA-binding domain led to the identification of three distinct regions, termed repressor domains I, II, and III, that possessed intrinsic repressor activity (Fig. 1). Finally, overexpression of the C-terminal domain of N-CoR lacking repression domains I-III relieves repression by the unli-

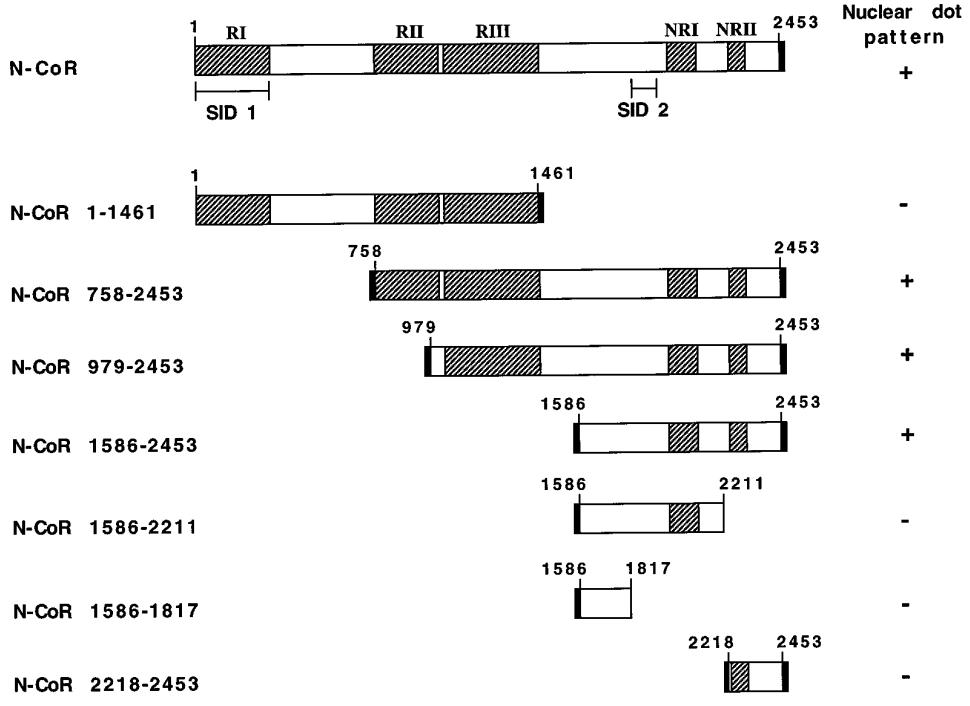

Fig. 1. Schematic Diagram of N-CoR and N-CoR Deletion Mutants Used in These Studies

Domains that transfer repression to the GAL4 DNA-binding domain are indicated as RI, RII, and RIII. Regions that mediate interactions with mSin3 are indicated as SID1 and SID2. Regions that mediate nuclear receptor interactions are labeled NRI and NRII. The *solid box* at the amino terminus of N-CoR deletion mutants indicates the presence of an engineered nuclear localization signal. The *solid box* at the carboxy terminus of N-CoR indicates an epitope (FLAG) tag that was used to facilitate immunolocalization experiments. Plus and minus signs at the *right* of the figure indicate whether or not the corresponding proteins localized to discrete dot structures.

ganded thyroid hormone receptor (16). Based on these observations, N-CoR was proposed to function as a nuclear receptor corepressor (12, 13).

In addition to N-CoR, expression cloning studies led to the identification of a related protein, termed SMRT (silencing mediator of retinoic acid and thyroid hormone receptors) (17, 18). Like N-CoR, SMRT interacts with the unliganded thyroid hormone and retinoic acid receptors via conserved nuclear receptor interaction domains and strongly represses transcription of promoters containing GAL4-binding sites when linked to the DNA-binding domain of GAL4. Intriguingly, SMRT appears to lack regions homologous to N-CoR repression domains I and II, but contains an N-terminal region with significant sequence similarity to N-CoR repression domain III.

Recent investigations into the mechanisms by which N-CoR might exert repressive effects have suggested a link with members of the Sin3 family of transcriptional corepressors (34a). Sin3 was initially identified by genetic screens for suppresser mutations of a Swi 5 defect in yeast (19, 20). Mammalian homologs of Sin3 were subsequently discovered in the course of investigating mechanisms responsible for transcriptional repression by bHLHZ proteins of the Mad family (21, 22). Members of the Mad family, which are believed to be involved in the induction of terminal differentiation in a wide range of cell types, have previously been demonstrated to act as transcriptional repressors upon heterodimerization with Max (23–29). The Mad proteins contain an N-terminal region that is required for transcriptional repression. Utilizing this repression domain to identify interacting proteins, mammalian homologs of Sin3 (mSin3A and mSin3B) were identified (21, 22).

Genetic studies in yeast have linked transcriptional repression by Sin3 to the global transcriptional regulator RPD3 (30–33). A human homolog of RPD3, hdac1, was isolated by virtue of its interaction with trapoxin, a histone deacetylase inhibitor (34). These observations led to the demonstration that RPD3 and hdac1 both posses histone deacetylase activity, suggesting a direct link between histone deacetylation and transcriptional repression.

Based on the parallels between transcriptional repression by nuclear receptors and Mad/Max heterodimers, studies have been performed to determine whether there might be functional interactions between mSin3, N-CoR, and RPD3. Consistent with this possibility, mSin3 and N-CoR have recently been found to directly interact *in vitro* (34a). Two Sin interaction domains within N-CoR were demonstrated to be capable of mediating these interactions, an N-terminal SIN interaction domain (SID 1) extending from amino acids 254 to amino acid 312 in repression domain I and a second domain (SID 2), extending from amino acids 1829 to 1940, in the C terminus of N-CoR (Fig. 1 and Ref. 34a). Evidence that the interactions between N-CoR and mSin3A/B are relevant to the repressive activities by nuclear receptors was provided by microinjection experiments in which cells were injected with antibodies directed against either N-CoR or the murine Sin3 homologs (34a). These experiments provided evidence that mSin3A/B and N-CoR were each required for transcriptional repression by nuclear receptors and by Mad.

A potential role for RPD3 in repression events was suggested by immunoprecipitation experiments, in which anti-Sin3A/B antibodies coprecipitated RPD3 (R. Eisenman, personal communication). Furthermore, microinjection of anti-RPD3 antibodies relieved repression by the nuclear receptor ligand-binding domain and by Mad (34a). In concert, these observations suggested the existence of a corepressor complex containing N-CoR, mSin3A/B, and RPD3 that is required for transcriptional repression by at least two distinct families of transcription factors.

To further examine the possibility that N-CoR, Sin3A/B, and RPD3 function as components of a corepressor complex in cells, we have performed a series of experiments examining the functional properties of N-CoR deletion mutants and the subnuclear localization of N-CoR and RPD3 using specific antibodies. Paradoxically, marked overexpression of N-CoR relieves, rather than potentiates, the repressive effects of the unliganded retinoic acid receptor. These observations suggest that alterations in the stoichiometry of corepressor function result in inhibition of the repressor complex. At low levels of expression, N-CoR is widely distributed throughout the nucleus in a nonuniform pattern, while at high levels of expression, an additional subpopulation of N-CoR becomes localized to multiple discrete dot structures. This discrete pattern of localization requires the presence of the N-CoR C terminus. RPD3 is also widely distributed throughout the nucleus in a nonuniform pattern. RPD3 and N-CoR appear to partially colocalize, consistent with the possibility that they are involved in the formation of a corepressor complex. RPD3 does not colocalize with N-CoR in the dot structures, however, and RPD3 and N-CoR are independently distributed throughout much of the nucleus. These observations suggest that the interactions between N-CoR and RPD3 may be dynamically regulated and are consistent with biochemical studies of histone deacetylases in yeast that suggest functionally distinct complexes.

RESULTS

A schematic diagram of N-CoR and a series of N-CoR deletion mutants used in these studies are illustrated in Fig. 1. Domains involved in transcriptional repression, nuclear receptor interaction, and mSin3A/B interaction are indicated. In the case of deletion mutants lacking the extreme amino terminus, a nuclear localization signal was incorporated into the expression vector to ensure appropriate targeting of N-CoR derivatives to the nucleus. In addition, to facilitate immu-

nolocalization of these various derivatives, an epitope (FLAG)-tag was engineered into the carboxy terminus.

Functional analysis of N-CoR and N-CoR deletion mutants was initially performed by assessing their effects on the transcriptional properties of retinoid acid receptor (RAR)/retinoid X receptor (RXR) heterodimers bound to the DR1 element present in the CRBPII promoter (Fig. 2). On this element, the RAR has been shown to constitutively repress transcription by RXR in

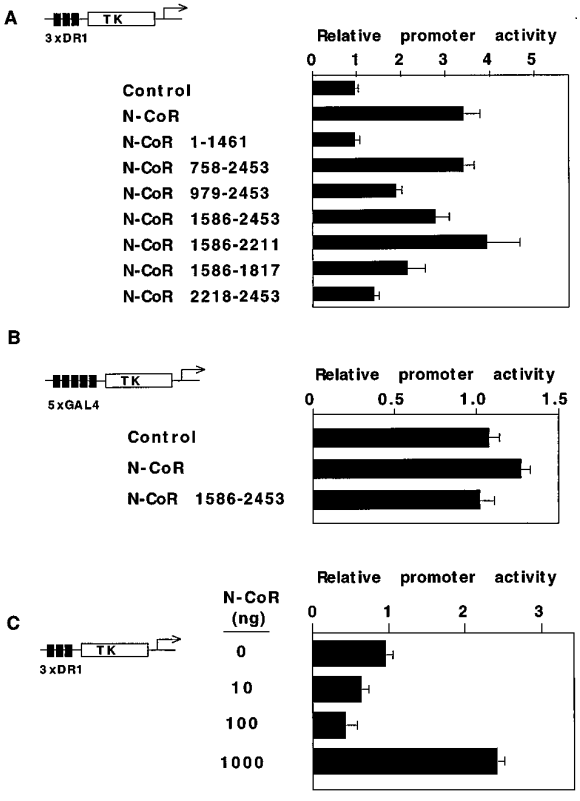

Fig. 2. Effects of Overexpression of Full-Length N-CoR and N-CoR Deletion Mutants on Transcriptional Activity of a DR1-Containing Promoter

A, Effects of N-CoR and N-CoR deletion mutants on transcription from the HSV-TK promoter containing three copies of the DR1 response element from the CRBPII gene. The 3xDR1 TK luciferase reporter plasmid was transiently transfected with 1 μg of expression vector for each of the indicated N-CoR deletion mutants, as described in *Materials and Methods*. Reporter gene activity was determined 24 h later. Promoter activity in the presence of overexpressed N-CoR or N-CoR deletion mutants is expressed relative to the activity of the 3x/DR1-TK-luciferase reporter gene alone. B, Effects of N-CoR and N-CoR 1586–2453 overexpression on transcription from the HSV-TK promoter containing five copies of a binding site for the GAL4 DNA-binding domain. Cells were transfected with 1 μg of the 5xGAL4-TK reporter and 1 μg of the N-CoR or N-CoR 1586–2453 expression plasmids and assayed for luciferase activity 24 h later. C, Influence of the levels of N-CoR expression on transcriptional activity of the 3xDR1-TK promoter. Cells were cotransfected with the indicated amounts of N-CoR expression plasmid and 100 ng of a CBP expression plasmid to increase basal levels of promoter activity.

CV1 cells (35, 36). We have previously demonstrated that N-CoR remains bound to RAR on this element, even in the presence of retinoic acid (12). As illustrated in Fig. 2A, several deletion mutants of N-CoR resulted in ligand-independent derepression of the RAR/RXR heterodimer on the DR1 element, consistent with the possibility that they functioned as dominant negative inhibitors of endogenous N-CoR function. Surprisingly, however, overexpression of the full-length N-CoR protein, rather than potentiating transcriptional repression, also resulted in increased levels of transcription from the DR1 element (Fig. 2A). This effect was dependent on the presence of the DR1 elements because overexpression of N-CoR or N-CoR 1586–2453 had no effect on the thymidine kinase (TK) promoter containing GAL4-binding sites (Fig. 2B). One possible interpretation of this paradoxical effect of N-CoR might be that at high levels of expression, N-CoR titrates out additional components required for the function of a corepressor complex. To assess this possiblility, transcriptional activity of the DR1-containing promoter was evaluated over a wide range of N-CoR expression by transfecting between 10 ng to 5 μg of N-CoR expression plasmid. At low levels of N-CoR expression (10–100 ng expression plasmid per well), transcription from the DR1-containing promoter was reduced, while at 1–5 μg of N-CoR expression plasmid, transcription was significantly increased (Figs. 2C and 3A). Indeed, when 1–5 μg of N-CoR expression plasmids were transfected, there was not only an increase in basal expression from the DR1-containing promoter, but the transcriptional response to the RAR-specific ligand, TTNPB, was also significantly increased (Fig. 3A). When evaluated on a promoter containing two copies of the DR5 element present in the RARβ2 promoter, transfection of 1–5 μg of N-CoR expression plasmid similarly resulted in a marked increase in promoter activity in the absence of ligand, consistent with relief of ligand-independent repression (Fig. 3B).

These observations raised the question of whether N-CoR might be expressed at different levels in specific cell types or during programs of differentiation. To examine this question, levels of N-CoR expression were evaluated in embryonic stem (ES) cells by Far-Western and Western blot experiments (Fig. 4). Undifferentiated ES cells exhibited very high levels of N-CoR. Intriguingly, N-CoR levels were markedly down-regulated during the *in vitro* differentiation of ES cells into embryoid bodies after removal of leukemia-inhibiting factor (LIF). These observations indicate that N-CoR levels can be dynamically regulated in cells.

To evaluate the subcellular distribution of N-CoR, indirect immunofluorescence microscopy was performed using cells transiently expressing epitope-tagged N-CoR. As illustrated in Fig. 4A, transient expression of epitope-tagged N-CoR in CV1 cells revealed a finely granular nuclear pattern of staining that was excluded from the nucleolus. In many cells, N-CoR staining was also observed in a number of

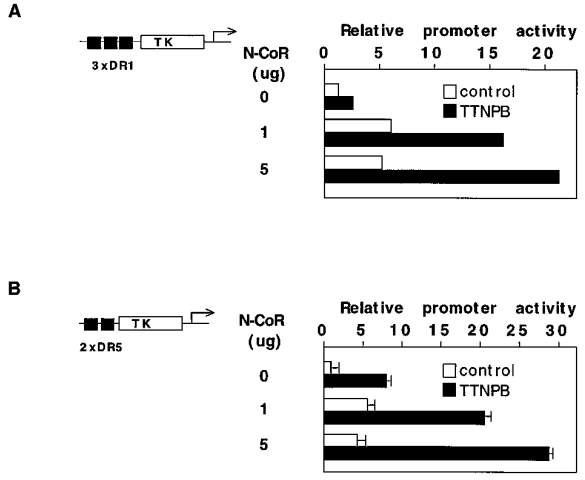

Fig. 3. Effects of N-CoR Overexpression on Ligand-Dependent Transcription from DR1- and DR5-Containing Promoters

Panel A, CV1 cells were transfected with 1 μg of the 3xDR1-TK promoter and the indicated amounts of N-CoR expression plasmid. Cells were treated with the RAR-specific ligand, TTNPB, as indicated and harvested for luciferase activity 24 h later. B, CV-1 cells were transfected as in panel A, except that the HSV-TK reporter gene contained two copies of the DR5 response element from the RARβ_2 promoter.

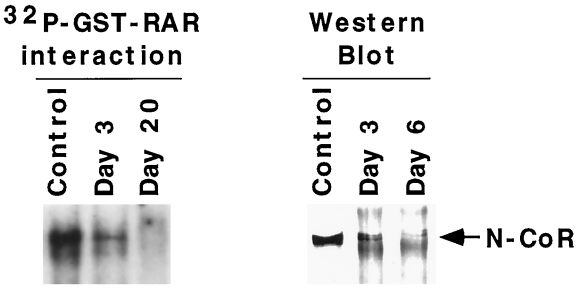

Fig. 4. Expression of N-CoR in Undifferentiated ES Cells and Embryoid Bodies

D3 ES cells were maintained in LIF (control), or in media lacking LIF for 3 and 20 days, resulting in the formation of embryoid bodies. Whole-cell extracts were prepared and N-CoR was captured on a GST-RAR affinity matrix. Proteins bound to RAR were resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and detected using either [^{32}P]GST-RAR (*left*) or a specific anti N-CoR antisera (*right*).

discrete dot structures. The accumulation of N-CoR in these structures appears to be a consequence of high levels of N-CoR expression, as parallel experiments using antibodies directed against the N- or C-terminal regions of the endogenous N-CoR in nontransfected cells exhibited the identical granular staining pattern as exogenously expressed N-CoR, but did not stain the discrete dot structures (data not shown). Furthermore, increased levels of N-CoR expression, achieved by transfection of increasing amounts of N-CoR expression plasmid, were correlated with an increase in the number and size of these dot structures (Fig. 5, panels B-D). A similar pattern of N-CoR staining was observed in HeLa cells and P19 cells (data not shown), indicating that localization of N-CoR into these discrete structures was not cell type-specific. Treatment of cells with retinoic acid did not alter the staining pattern of N-CoR (data not shown).

We next examined the staining patterns of the series of N-CoR deletion mutants evaluated functionally in Fig. 2. As illustrated in Fig. 6B, the N-terminal region of N-CoR (amino acids 1–1461) containing repressor domains 1, 2, and 3 was localized to the nucleus in a finely granular pattern, but failed to stain the dot-like structures observed for full-length N-CoR. In contrast, a construct containing C-terminal amino acids from 758 to 2453 exhibited both the granular and dot-like patterns of staining (Fig. 5C). Additional amino-terminal deletion mutants beginning at amino acids 979 and 1586 also exhibited both granular and dot-like patterns of staining (Fig. 6, D and E). A further N-terminal deletion to amino acid 2218 resulted in a diffuse pattern of staining in both the cytoplasm and nucleus. An internal region of N-CoR extending from 1586 to 2211 also failed to exhibit a dot-like staining pattern. Most of the N-CoR deletion mutants that accumulated in nuclear dot structures were also observed to relieve repression. However, relief of repression was also observed for some N-CoR derivatives that did not localize to nuclear dot structures. For example, N-CoR 1586–2211 did not accumulate in dot structures but was effective at relieving repression. This may reflect the fact that N-CoR 4586–2211 retains one of the nuclear receptor interaction domains (NRI) and would be expected to compete with endogenous N-CoR for interaction with RAR.

We next evaluated the subcellular localization of RPD3. As illustrated in Fig. 7A, indirect immunofluoresence staining of RPD3 exhibited an exclusive nuclear staining pattern. RPD3 was excluded from the nucleolus, but was otherwise widely distributed in a nonuniform pattern. To determine whether RPD3 and N-CoR were colocalized, confocal laser scanning microscopy was performed. At high image magnification, RPD3 was localized to many small, diffuse clusters (Fig. 7A). In addition to the discrete dot-like structures, N-CoR was also localized to diffuse clusters, although this pattern was somewhat less distinct than that observed for RPD3 (Fig. 7C). Examination of cells exhibiting low, moderate, and high levels of transfected N-CoR expression revealed similar patterns of staining, with the exception that the discrete dot pattern was only observed at higher levels of N-CoR expression (data not shown). Upon merging the images obtained for N-CoR and RPD3 within the same optical section, some colocalization of the two antibodies was observed, as evidenced by the yellow staining pattern (Fig. 7B). A significant fraction of the staining for the two proteins did not colocalize, however, and several regions were observed that stained brightly for RPD3, but not N-CoR, or brightly for N-CoR, but not RPD3. Thus, although these observations are consistent with

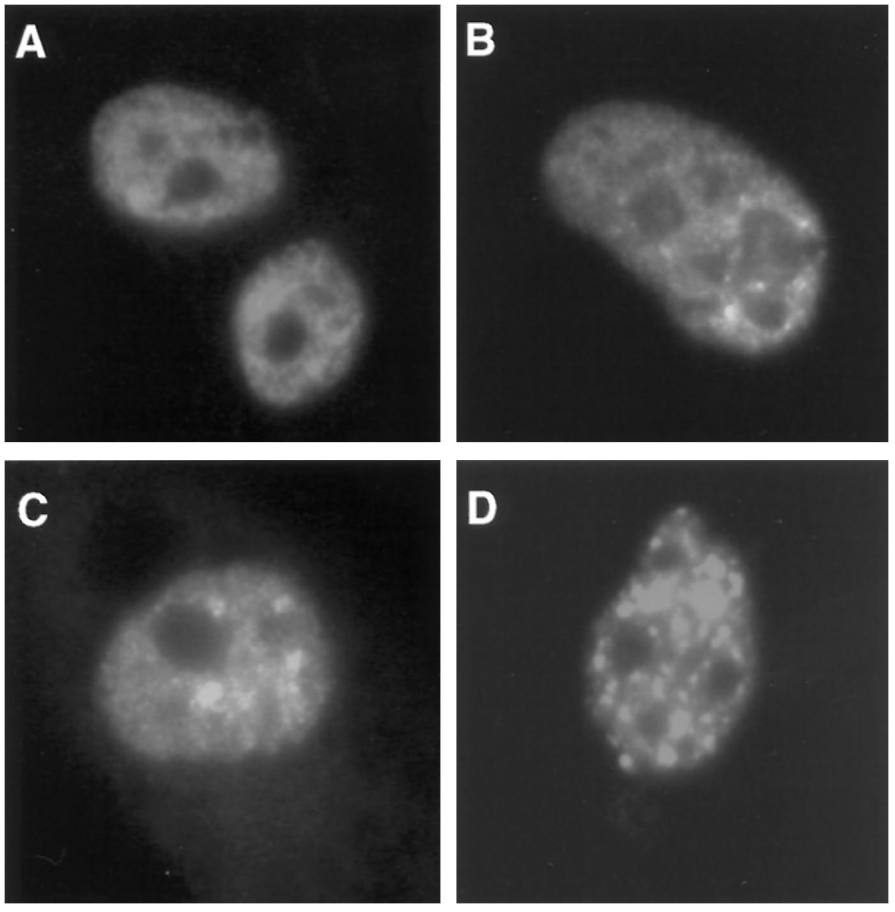

Fig. 5. Subcellular Distribution of N-CoR in CV-1 Cells

CV-1 cells were plated on glass coverslips and transfected with increasing amounts of a vector directing the expression of full-length, epitope-tagged N-CoR (corresponding to 0.1 to 2.2 μg DNA/10 cm^2 of plating surface). Twenty four hours later, the cells were fixed and prepared for indirect immunofluorescence labeling using the anti-epitope antibody as described in *Materials and Methods*. Panels A through D are images of representative cells that express progressively higher levels of N-CoR in dot structures. The immunostaining patterns were exclusively nuclear, and the cell bodies are not apparent. (Magnification of original images, 100×).

biochemical experiments indicating that RPD3 and N-CoR interact, they suggest that a substantial fraction of these proteins reside in separate compartments within the nucleus.

DISCUSSION

Transcriptional repression has been demonstrated to be an important strategy for the regulation of growth, development, and homeostasis in several experimental systems (37–42). In the cases of the retinoic acid and thyroid hormone receptors, the specific biological roles of their transcriptional repression functions are not well understood. However, *cErb-A*, the oncogenic counterpart of the thyroid hormone receptor, is thought to collaborate with other oncogenes and promote uncontrolled cell growth by virtue of its activity as a constitutive transcriptional repressor (5, 7, 43, 44). In contrast, transcriptional repression by Mad is correlated with growth arrest and the establishment of differentiated cell phenotypes (23–29). Thus, the repressive functions of unliganded thyroid hormone and retinoic acid receptors and members of the Mad family can be considered to have opposing effects on programs of growth and differentiation and are therefore likely to regulate distinct sets of genes.

Investigation of the mechanisms responsible for transcriptional repression by the retinoic acid and thyroid hormone receptors led to the identification of N-CoR and the related factor SMRT, which were subsequently demonstrated to mediate transcriptional repression by v-Erb A and Rev-erb (12, 13, 15, 18). In contrast, mSin3A/B were identified as putative corepressors of Mad (21, 22). Protein-protein interaction assays and coimmunoprecipitation studies have recently demonstrated that N-CoR and mSin3 A/B are components of a corepressor complex that is required

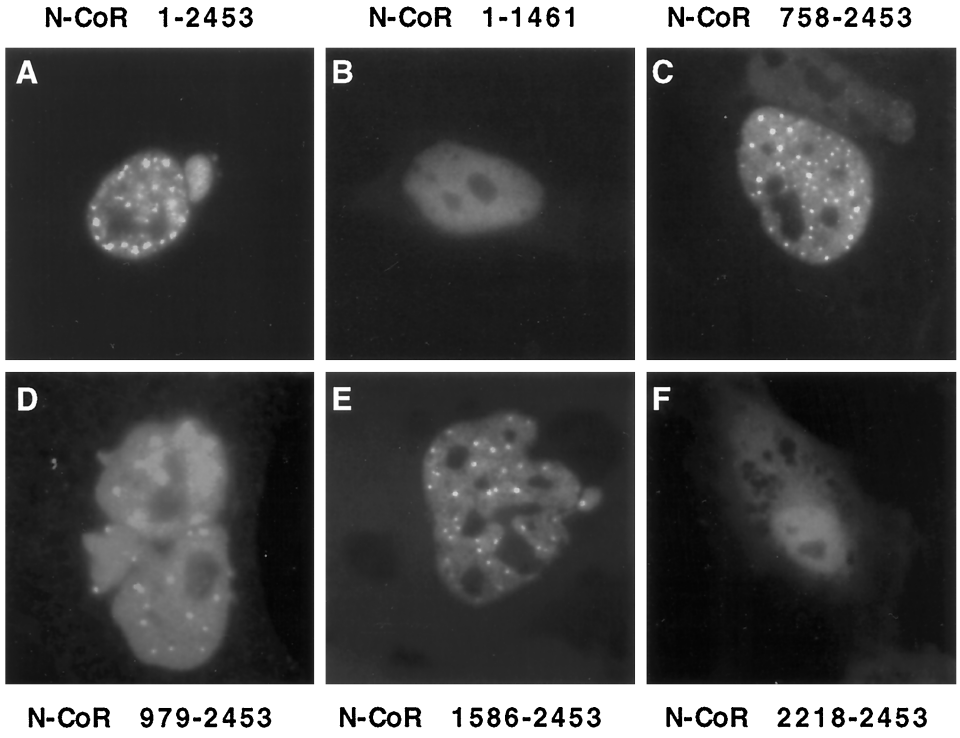

Fig. 6. The C-Terminal Domain of N-CoR Is Required for Localization to Discrete Nuclear Structures

CV-1 cells were plated on glass coverslips and transfected with vectors directing the expression of the indicated epitope-tagged N-CoR deletion mutants. Twenty four hours later, the cells were fixed and prepared for indirect immunofluorescence labeling. Representative cells are shown. Staining is exclusively nuclear in panels A-E. Some extranuclear staining is also observed in panel F. (Magnification of original images, 100×)

for the repression functions of both Mad and unliganded RAR and thyroid hormone receptor (34a). Intriguingly, Sin3 has been demonstrated previously to inhibit the activity of the progesterone receptor in yeast, suggesting that it may play a more general role in regulating nuclear receptor function (45). Although direct interactions have not yet been established, immunoprecipitation of mSin3 A/B or of N-CoR from cells coprecipitates the histone deacetylase, RPD3. RPD3 appears to be required for repression by unliganded nuclear receptors and Mad because microinjection of anti-RPD3 antibodies into cells reverses their repressive effects (34a).

The link between histone deacetylases and corepressor proteins that directly interact with Mad and unliganded retinoic acid and thyroid hormone receptors suggests an attractive mechanism by which these factors repress the transcription of target genes. It has been well established that nucleosomes play important roles in both positive and negative regulation of transcription (reviewed in Refs. 39, 46, and 47). The dynamic effects of nucleosomes on transcription have been suggested to be due, in part, to the acetylation of lysine residues present in the amino-terminal ends of the core histones (46). Numerous studies have demonstrated that nucleosomes containing hyperacetylated histones are colocalized with actively transcribed regions of the genome (48–50). Conversely, histone hypoacetylation is associated with heterochromatic regions of DNA that are transcriptionally silent (51, 52). The mechanisms by which the state of histone acetylation influences transcription have not been established, but acetylation of the N-terminal tails of histones H3 and H4 appears to facilitate the association of other transcription factors with nucleosomal DNA (53).

The acetylated state of the core histones is thought to be determined by the competing activities of histone acetylases and deacetylases. In this regard it is intriguing to note that transcriptional activation by liganded nuclear receptors has been demonstrated to require cAMP response element binding protein (CBP) and/or p300 (54–57). CBP and p300 each posses intrinsic histone acetylase activity (58, 59) and can associate with p/CAF, which also exhibits histone acetylase activity (60). These observations suggest a highly symmetric model in which transcriptional repression is mediated by a complex containing N-CoR, mSin3, and RPD3, which effects local histone deacetylation. Upon binding of activating ligands, this deacetylase-containing complex dissociates from nuclear receptors and is replaced by a coactivator complex containing CBP/p300 and p/CAF, which acetylates histones and facilitates entry of core transcription factors.

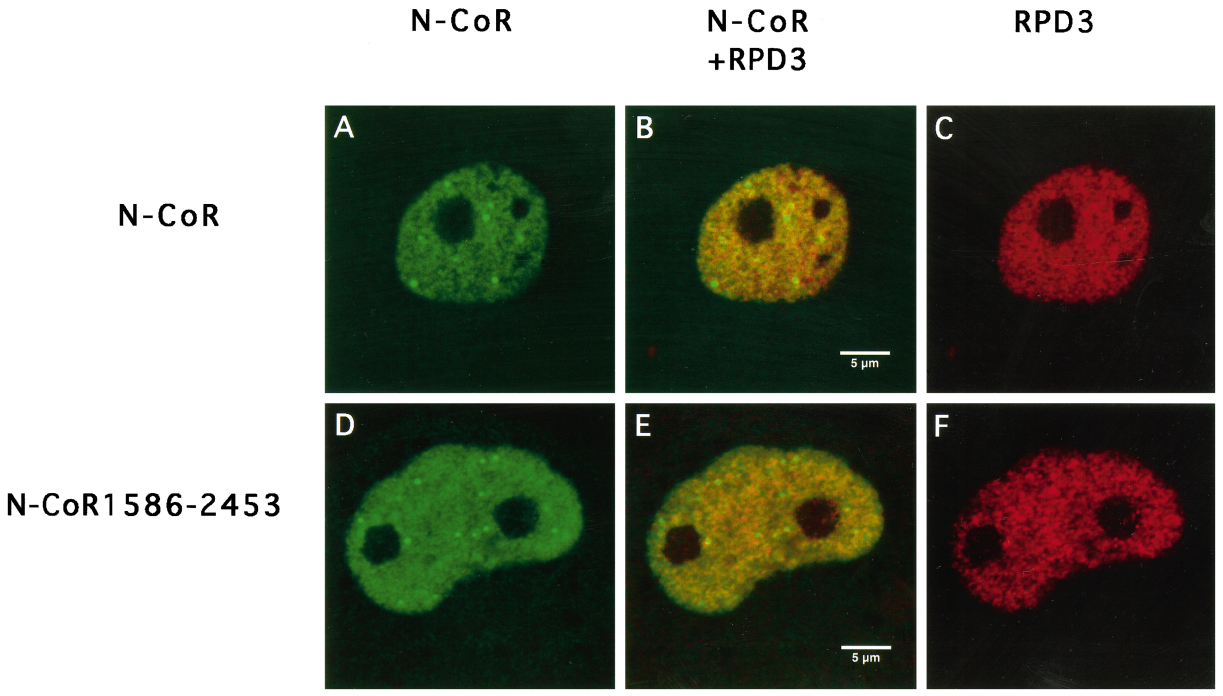

Fig. 7. Colocalization of RPD3 and N-CoR in the Nucleus

CV-1 cells were plated on glass coverslips and transfected with epitope-tagged N-CoR (A, B, C) or N-CoR1586-2453 (D, E, F). Cells were harvested 24 h later and immunostained with anti-RPD3 antisera (Cy3) and anti-epitope (FLAG) antibody (FITC). Images were collected using a Bio-Rad (Hemel Hempstead, UK) confocal imaging system linked to an Axiovert microscope (Carl Zeiss, Thornwood, NY) at an optical magnification of 63×. The RPD3 signal alone is illustrated in panels A and D. The N-CoR signal alone from the same optical section is illustrated in panels C and F. The sections are merged in panels B and E. Regions in which N-CoR and RPD3 colocalize appear *yellow*.

In the present study, we have evaluated the consequences of overexpression of N-CoR and N-CoR deletion mutants and have examined the intracellular distributions of N-CoR and RPD3 in the cell. At low levels of expression, both N-CoR and RPD3 are distributed in the nucleus in a nonuniform, finely granular pattern. Based on images obtained using confocal laser scanning microscopy, a fraction of N-CoR and RPD3 appear to be colocalized, consistent with biochemical evidence for their presence in a corepressor complex. However, a significant fraction of the two proteins also appears to be independently distributed. These observations suggest heterogeneity in the composition of corepressor complexes containing RPD3 and/or the possibility that the formation of mSin3/N-CoR/RPD3 is dynamically regulated. Biochemical evidence for heterogeneity in histone deacetylase complexes has been obtained in yeast, in which two deacetylase complexes have been identified, termed HDA and HDB, that exhibit different sensitivities to deacetylase inhibitors (61).

Overexpression of several regions of N-CoR involved in nuclear receptor or mSin3 interaction resulted in derepression of RAR/RXR heterodimers bound to a DR1 element, consistent with the possibility that they serve as dominant negative inhibitors of endogenous N-CoR activity. Surprisingly, marked overexpression of N-CoR relieved receptor-dependent repression. These observations raised the possibility that active repression by N-CoR might require a specific stoichiometry of corepressor components, and that overexpression of N-CoR might cause redistribution of some of these components away from the target promoter. Localization of N-CoR by indirect immunofluorescence revealed that, when expressed at high levels, a significant fraction of N-CoR was redistributed to discrete dot structures within the nucleus that were not observed for endogenous N-CoR. Localization of overexpressed N-CoR to these structures required an extended region of the N-CoR C terminus, containing the nuclear receptor and C-terminal mSin3 interaction domains. The redistribution of N-CoR to these discrete structures is consistent with the possibility that disruption of corepressor complexes accounts for the relief of repression by unliganded RAR/RXR heterodimers observed after N-CoR overexpression. RPD3 was not colocalized to these structures, consistent with the lack of evidence for a direct interaction between RPD3 and N-CoR or mSin3. Additional studies localizing nuclear receptors, mSin3, and other putative components of the corepressor complex should help resolve the possible function of these discrete structures.

Studies of N-CoR expression in ES cells revealed very high levels in undifferentiated cells and marked down-regulation during their differentation into embry-

oid bodies. These results are consistent with *in situ* hybridization studies that indicate very high levels of N-CoR mRNA in embryonic tissues. These levels markedly decline during later stages of development and reach much lower levels in adult tissues (M. G. Rosenfeld, unpublished observation). Thus, N-CoR levels appear to be dynamically regulated during development and differentiation. Such changes in N-CoR expression are likely to have significant effects on repression mediated by nuclear receptors and Mad. Intriguingly, the RAR/RXR heterodimer does not constitutively repress transcription from DR1-containing promoters in several ES cell lines (62). These observations raise the possibility that high levels of N-CoR in ES cells may result in derepression, rather than enhanced repression, if other components of the corepressor complex are limiting. It will be of considerable interest to determine whether the marked variations in levels of N-CoR expression result in significant alterations in patterns of transcriptional repression that might have important consequences for normal programs of growth and development.

MATERIALS AND METHODS

Construction of N-CoR Deletion Mutants

Two oligonucleotides corresponding to two copies of the FLAG sequence, including a *Bam*HI site (5′) and a *Not*I site (3′) for cloning, were synthesized (sense: 5′-GCGGGATCCGACTACAAGGACGACGATGACAAGGACTACAAGGACGACGATGACAA-GTGAGCGGCCGCGGGCCCAGAG-3′) annealed, digested with *Bam*HI and *Not*I, fused in frame at the C terminus of N-CoR, and placed under the control of a cytomegalovirus transcription unit. Two oligonucleotides corresponding to a nuclear localization signal (NLS) (PKKKRK) and an ATG start site (sense: 5′-AGCTTGACCACCATGGGTACCATGCCAAAGAAGAAGAGGAAGGTAC) were annealed and fused at the *Hin*dIII-*Kpn*I sites of the multiple cloning site of pcDNA 3 (Invitrogen Carlsbad, CA) creating an open reading frame polycloning site (pcDNA3-NLS). N-CoR 758-2453 was constructed by fusing the *Xho*I-*Not*I fragment of N-CoR with the pcDNA3-NLS vector digested in the same way. N-CoR 979-2453 was constructed by fusing the *Nsi*I-*Not*I fragment of N-CoR with the pcDNA3-NLS vector digested in the same way. N-CoR 1586–2453 was constructed by fusing the *Bam*HI fragment (4871–7480) of N-CoR with the pcDNA3-NLS vector digested in the same way and screened for orientation. The FLAG epitope was put in place by replacing the *Hin*dIII-*Not*I fragment with the corresponding fragment cut out from full-length N-CoR/FLAG. N-CoR 2218–2453 was constructed by digesting N-CoR with *Hin*dIII-*Not*I, filled and fused with Rexp, digested with *Bam*HI, and filled and placed under the control of a rous sarcoma virus transcription unit. A nuclear localization sequence was engineered by synthesizing two oligonucleotides encoding the NLS and fused in frame at the *Nco*I site (6766). N-CoR 1–1461 was constructed by digesting N-CoR with *Sac*I and *Not*I and fused with pcDNA3. A FLAG epitope was constructed by using oligonucleotides encoding to two copies of the FLAG sequence and fused in frame at the C terminus. N-CoR 1586–1817 were constructed by digesting N-CoR 1586–2453 with *Apa*I and religating the vector. N-CoR 1586–2211 were constructed by digesting N-CoR with *Hin*dIII, filled, and recut with *Bam*HI. The fragment was ligated into pcDNA3-NLS digested with *Bam*HI and *Eco*RV. A FLAG epitope was put in frame at the N terminus of N-CoR 1586–1817 and N-CoR 1586–2211 constructs by using oligonucleotides encoding two copies of the FLAG sequence, fused in frame at the *Bam*HI site directly after the NLS signal.

Transient Transfection Assays

Transfections were performed in six-well plates by the calcium phosphate method as described previously (34). If not otherwise indicated, each well was transfected with 1 μg reporter plasmid and 1 μg N-CoR constructs. The precipitate was washed away with PBS after 12 h, and the media was replaced. The cells were harvested after an additional 24 h and assayed for luciferase activity.

Indirect Immunofluorescence Analysis

Cells were grown on coverslips in 6-cm dishes and were transiently transfected with N-CoR constructs as indicated. Cells were washed after 12 h, after which the media were replaced, and the cells were incubated for an additional 24 h. Cells were then fixed in 2% paraformaldehyde in PBS for 45 min at room temperature, washed in PBS, and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Monoclonal FLAG antibody M2 (Kodak, New Haven, CT) (50 μg/ml in PBS, 1% BSA) was added for 1 h at room temperature. For double-staining, cells were incubated with monoclonal FLAG antibody M2 (50 μg/ml) plus rabbit-mRPD3 antisera (1:50 dilution in PBS, 1% BSA) for 1 h, followed by five washes in PBS, 0.1% BSA. Fluorochrome-conjugated secondary antibody (anti-mouse fluorescein isothiocyante (FITC) affinipure F (ab′)$_2$ goat IgG diluted 1:150 was applied for 60 min followed by five washes. Double-stained cells were incubated for 60 min with fluorochrome-conjugated secondary antibody (anti-mouse FITC affinipure F (ab′)$_2$ goat IgG plus biotinylated anti-rabbit goat antisera (diluted 1:150), washed five times in PBS-0.1% BSA, and incubated with Cy3-conjugated streptavidin (diluted 1:1000), followed by five washes in PBS-0.1% BSA.

Confocal Laser Scanning Microscopy

Fluorescent sections were imaged using a Bio-Rad MRC600 confocal microscope (Hemel Hempstead, UK) equipped with a krypton/argon laser and coupled to a Zeiss Axiovert 135 M microscope (Carl Zeiss, Thornwood, NY) as described (63).

Analysis of N-CoR in ES Cells

D_3 ES cells were maintained in an undifferentiated state by culture in the presence of leukemia-inhibitory factor (LIF). To induce embryoid body formation, ES cells were transferred to LIF-deficient media and plated at 1×10^4 cells/ml. Embryoid bodies formed over the next 5–12 days and exhibited morphological evidence of hematopoietic development and the development of contractile cells. Whole-cell extracts were prepared from undifferentiated ES cells and embryoid bodies as described by Halachmi *et al.* (64). N-CoR was purified on a glutathione-*S*-transferase (GST)-RAR affinity matrix, resolved by SDS-PAGE, and transferred to nitrocellulose membranes as described by Kurokawa *et al.* (12). N-CoR was detected using [^{32}P]GST-RAR or anti N-CoR antisera as described (12).

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