The Nuclear Receptor Co-repressor (N-CoR) Utilizes Repression Domains I and III for Interaction and Co-repression with ETO*

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The acute human leukemias are associated with the presence of chimeric gene products that arise from spontaneous chromosomal translocations. The t(8;21) translocation gene product led to the discovery of the Eight Twenty-One (ETO) gene. When fused to RUNXI, ETO is thought to mediate the formation of a repressive complex at RUNXI-dependent genes. ETO has also been found to act as a co-repressor of the promyelocytic zinc finger and Bcl-6 oncoproteins, suggesting that it may play a common role as a transcriptional co-repressor leading to human disease. An analysis of ETO-mediated repression revealed that one of the key binding partners of ETO is the nuclear receptor co-repressor (N-CoR). It is shown that two highly conserved domains of ETO interact with repression domains I and III of N-CoR. One of the ETO domains displays significant homology to Drosophila TAF₁₁₀, whereas the other is a predicted zinc binding motif that engages a conserved PPLX motif in repression domain III of N-CoR. Together, these domains of ETO cooperate in repression with N-CoR and the binding sites in N-CoR overlap with those for other repressive factors. Thus, ETO has the potential to participate in a number of repressive complexes, which can be distinguished by their binding partners and target genes.

The ability of cells to adjust to different internal and external signals is crucial in development, growth, and homeostasis of organisms (1). One level of this regulation takes place in the nucleus where transcription factors are recruited to their target genes through direct interaction with their cognate DNA binding sequences. A variety of cofactors senses cellular signals and modulates the transcriptional response by direct interaction with DNA-bound transcription factors (2, 3). These cofactors recruit multiprotein complexes to the promoters of genes and remodel chromatin, changing the accessibility of the promoter to the transcription machinery (2, 4, 5). Co-activators like CREB¹-binding protein/p300 (6), GCN5 (7), or PCAF (8) acetylate the N-terminal tails of core histones, which facilitate access to a region of DNA to be transcribed by RNA polymerase. Conversely, co-repressor molecules, such as the nuclear receptor co-repressor (N-CoR) (9), recruit proteins that include histone deacetylases (HDACs), which remove acetyl groups from the histone tails and thereby repress transcription from specific genetic loci (2, 4, 10). N-CoR is one of a number of proteins (9, 11, 12) that form multiprotein complexes, which silence transcription through direct interaction with transcription factors bound to promoters and enhancers. The composition of these co-repressor complexes varies depending on the promoter and cellular context (2, 13). A number of human diseases have been linked to the misassociation of co-activators or co-repressors with transcription factors that alter gene expression patterns during development (14, 15).

The protein known as Eight Twenty-One (ETO, MTG8) was first identified as a component of the t(8;21)-chromosomal translocation gene product (16–18). Spontaneous translocation fuses a portion of the gene encoding the DNA-binding Runx domain of RUNXI (acute myeloid leukemia 1 (AML1), polyoma enhancer-binding protein αB, and core binding factor A2) in-frame with all but 18 nucleotides of the EETO gene, resulting in the AML1-ETO fusion protein with new transcriptional properties. This fusion protein is expressed in 12% human AML of the M2-subtype (16–18), and there is strong evidence that the EETO component of AML1-ETO interacts with a number of co-repressor complex proteins (e.g. N-CoR, Sin3, and several HDACs) (19–25). The targeted deletion of the eto gene causes defects in gut development in a mouse model (26), and overexpression of the Xenopus EETO homolog XETOR suggests a role for ETO in neuronal development (27). ETO has been shown to act as a co-repressor for the promyelocytic zinc finger protein (23) and also cooperates with GFI-1 and Atrophin-1 in transcriptional repression (28, 29). ETO can also act as a co-repressor for the Bcl-6 oncoprotein, indicative of a role in hematopoiesis (30). Because ETO is thought to interact with N-CoR, the silencing mediator for retinoid and thyroid hormone receptor (SMRT), Sin3, and HDACs 1, 2, and 3 (19, 24), it might act as a scaffold protein for different co-repressor complexes. Thus, the fusion of the RUNXI Runx domain to ETO would be predicted to suppress gene expression by delivering co-repressor proteins to RUNXI-dependent genes via direct interaction with ETO.

To elucidate the action of ETO in development and disease, a detailed analysis of repression by ETO and its subfragments has been conducted and the interaction with N-CoR has been defined. Two distinct conserved regions of ETO have been found to interact with different repression domains of N-CoR. One of these segments is a conserved domain with significant homology to Drosophila TAF₁₁₀ that interacts with repressed AML1, polyoma enhancer-binding protein αB, and core binding factor A2 (212-327-7222; E-mail: mwerner@portugal.rockefeller.edu.

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¹ The abbreviations used are: CREB, cyclic AMP-response element-binding protein; ETO, Eight Twenty-One; N-CoR, nuclear receptor co-repressor; HDAC, histone deacetylase; AML, acute myeloid leukemia; RI, repression domain I; RII, repression domain II; TAF, TBP-associated factor; luc, luciferase; tk, thymidine kinase; GST, glutathione S-transferase; NHR, Nervy homology domain; MYND, MTG8, Nervy, De-
RI and RIII of N-CoR Bind ETO

EXPERIMENTAL PROCEDURES

Plasmid and Cells—If not otherwise indicated, cloning was performed using PCR amplification with primer-introducing restriction sites. Expression plasmids for Gal4-ETO fusion proteins were generated by cloning in-frame to the Gal4 DNA-binding domain into the pBind Vector (Promega) using SacI and NotI restriction sites. Single amino acid changes were introduced using the QuikChange mutagenesis strategy (Invitrogen) according to the manufacturer’s instructions. For in vitro transcription/translation, the gene encoding N-CoR or ETO or its subdomains was inserted into pET-21 or pET-28b (Novagen). For expression in mammalian cells, the reading frames were inserted into pcDNA3 (Invitrogen). HEK-293 (CRL-1573, ATCC) and CV-1 (CCL-70, ATCC) cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine, and 110 mg/liter sodium pyruvate (Invitrogen).

Transfection Assay—A reporter vector containing five Gal4 DNA-binding domain-binding sites linked 5’ to the Herpesvirus thymidine kinase (tk) promoter, and the firefly luciferase gene (5’ Gal4-tk-luc) was used. As an internal control, a plasmid coding for β-galactosidase was co-transfected. Cells were plated in 12-well tissue culture dishes at a density of 5 × 10^4 cells/well. After 24 h, cells were transfected with FuGENE 6 (Roche Applied Science). Transcriptional activity was determined 48 h after transfection using a luciferase assay (Promega) and a tube luminometer (Berthold). Experiments were performed in duplicate.

FIG. 1. N- and C-terminal domains of ETO contribute to transcriptional repression. A, ETO-N and ETO-C were fused to the Gal4 DNA-binding domain and tested for their ability to repress transcription of a luciferase reporter. B, repression assay with 100 ng of 5×Gal4-tk-luc luciferase reporter gene co-transfected with 100 ng of ETO, ETO-N, or ETO-C into CV-1 cells (left) or HEK-293 cells (right). ETO-C has twice the repressive capacity of ETO-N, but the effect of the individual fragments is roughly additive with the full-length protein. As the expression control, a Western blot was performed using an anti-Gal4 antibody (sc-577, Santa Cruz Biotechnology). Transcriptional activity is given as relative light units (RLU) to the expression of the β-galactosidase gene. The total amount of DNA was adjusted with empty vector to a final amount of 400 ng/transfection. Fold repression is given as numbers over the columns and represents the RLU relative to the values gained with the full-length ETO construct. Error bars represent the mean ± S.D. of three experiments.

FIG. 2. Distinct repression domains of N-CoR interact with N- and C-terminal halves of ETO. A, schematic representation of N-CoR and the deletion mutants used in this study. RI and RIII are two of the repression domains (3). Black vertical bars represent the Sin3 interaction domains. B, the N-CoR-N interacts with ETO in a GST-ETO pull-down assay as described under “Experimental Procedures.” The input lanes represent 10% of the total labeled protein, and the control lanes contain GST only bound to glutathione-agarose. C, ETO-N or ETO NHR3/4 was fused to GST and probed for its ability to bind deletion mutants of N-CoR-N. It is shown that ETO-N binds to N-CoR RI and that ETO NHR3/4 interacts with N-CoR RIII. D, deletion analysis of N-CoR RI identified amino acids 254–407 as responsible for the interaction with ETO-N (left panel). Similarly, a minimal RIII domain of N-CoR from amino acids 950–1275 was found to interact with ETO NHR3/4 (right panel). Asterisks in the Input indicate the mobility of the expressed proteins.
cate and repeated at least three times. Luciferase values were normalized to β-galactosidase activity and expressed as relative light units or fold repression of the normalized luciferase activity.

In Vitro Protein Interaction Assay—Glutathione S-transferase (GST) fusion proteins were expressed using pGEX-4T1 (Amersham Biosciences). Plasmids were transformed into *Escherichia coli* BL21 strain, and single colonies were expanded and grown at 37 °C to an *A*<sub>r</sub><sub>600</sub> of 0.8 and subsequently induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside. After overnight growth at 20 °C, the cell pellet was collected and the GST protein was purified according to the manufacturer’s instructions. [35S]-Labeled proteins were produced by coupled in vitro transcription/translation in the presence of [35S]Met (with or without [35S]Cys as stated) using the rabbit reticulocyte TNT kit (Promega). Equal amounts of GST fusion protein were immobilized on glutathione-agarose beads (Sigma-Aldrich) and incubated with 10 μl of TNT reaction mixture in 300 μl of pull-down buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, 1 mM ZnSO<sub>4</sub>) at 4 °C for 1 h. The beads were washed four times using pull-down buffer. Afterward, the proteins were eluted by boiling the beads in 20 μl of SDS loading dye and subjected to SDS-PAGE. The gel was Coomassie Blue-stained for 20 min to check for equal protein loading. Subsequently, the gel was dried and analyzed after 24 h using a phosphorimaging screen.

**Immunoprecipitation and Western Blot**—Transiently transfected HEK-293 cells were harvested 48 h after transfection. The cells were lysed in immunoprecipitation assay buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% IGEPEAL (Sigma), 0.5% deoxycholate, 0.01% SDS, protease inhibitor (Roche Applied Science)). Antibody was added to the supernatant (anti-ETO sc-9737, anti-Myc 9E10, Santa Cruz Biotechnology) and incubated for 30 min. The sample was diluted 2-fold with washing buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% IGEPEAL), and 20 μl of protein A/G magnetic beads (New England Biolabs) were added and incubated for 1–2 h at 4 °C with rotation. The beads were washed twice with washing buffer and resuspended in 20 μl of SDS loading dye, boiled for 2 min, and subjected to SDS-PAGE. For Western blot to detect transfected Gal4 constructs, the cells were lysed with reporter lysis buffer (Promega) and SDS was added to a concentration of 0.5%. The cell extract was sonicated until it was no longer viscous and then boiled for 2 min, and subjected to SDS-PAGE. After SDS-PAGE, the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell) at 50 V for 2 h. The membrane was blocked for 1 h at room temperature with CaseinBlocker (Bio-Rad). Subsequently, the membranes were incubated with primary antibody for 1 h at 22 °C or overnight at 4 °C when the anti-Gal4 antibody (sc-577, Santa Cruz Biotechnology) was used. Afterward, the membranes were washed with phosphate-buffered saline containing 0.5% IGEPEAL. Membranes were incubated for 1 h with secondary antibody and washed as described above. The blots were developed using SuperSignalWest (34079, Pierce) according to the manufacturer’s instructions.

**RESULTS**

The N-terminal and the C-terminal Halves of ETO Mediate Transcriptional Repression—The human ETO gene family consists of three members, each of which contains four regions that display significant amino acid sequence similarity to *Drosophila* Nervy (Fig. 1) (31, 32). Nervy homology domain I (NHR1) is closely related in amino acid sequence to human TAF<sub>110</sub> and human TAF<sub>110</sub> and *Drosophila* TAF<sub>110</sub> (17, 33). NHR2 is proposed to mediate homodimerization (25) as well as heterodimerization with other ETO family members (34) and contributes to the interaction with the co-repressor Sin3 and to the HDACs (25). NHR3 interacts with the regulatory subunit of cAMP-dependent kinase protein kinase II (35). NHR4 contains a MYND-type (MTG8, Nervy, Deformed) zinc binding motif common to ETO and the *Drosophila* proteins Nervy and Deformed. The MYND motif is necessary but not sufficient for interaction with the transcriptional co-repressors N-CoR and SMRT (24, 25, 36). NHR3 and NHR4 are thought to be simultaneously required for interaction with N-CoR (36). Recent data also implicate a region N-terminal to NHR2 as also mediating an interaction with N-CoR (19).

To determine the domains of ETO that are responsible for mediating transcriptional repression, ETO was deconstructed and the capacity for ETO and its subfragments to repress transcription from a luciferase reporter was assessed. Both the N-terminal 305 amino acids (ETO-N, residues 1–305) and the C-terminal 304 amino acids of the protein (ETO-C, residues 300–604, see Fig. 1A) could individually suppress transcription from the reporter in green monkey CV-1 cells and HEK-293 cells. Full-length ETO repressed the reporter gene expression 10-fold in CV-1 cells, whereas the N- and C-terminal deletion fragments repressed expression 2- and 5-fold, respectively (Fig. 1B, left). This finding suggests that the overall repressive effect may be a sum of repressive events mediated by the N- and C-terminal halves of the protein. Similar levels of repression were observed for these ETO constructs in HEK-293 cells (Fig. 1B, right), suggesting that repression mediated by ETO in these two cells lines may occur by the same mechanism. As an expression control, a Western blot against the Gal4 part of the fusion protein was performed. Fig. 1B demonstrates that the repressive effect of ETO-N and ETO-C were not influenced by a higher level of expression by either half of ETO relative to the wild type. Thus, both halves of ETO possess repressive activity.

**Two Domains of ETO Bind to the Co-repressor N-CoR**—To investigate the origin of the repressive effect mediated by ETO, the ability of N-CoR to interact with ETO and its subfragments...
was analyzed. A GST fusion protein of full-length ETO was used as bait in a GST pull-down assay with the in vitro translated 35S-labeled N-CoR or its subdomains. An N-terminal fragment of N-CoR (N-CoR-N, residues 1–1507) interacted with GST-ETO, whereas a C-terminal deletion mutant encompassing residues 1300–2453 (N-CoR-C) did not (Fig. 2B). The interaction between ETO and N-CoR-N was further dissected with the N- and C-terminal deletions of ETO, revealing that ETO-N interacts with the N-CoR repression domain I (RI, residues 1–407) and that the C-terminal NHR3 and NHR4 domains of ETO (NHR3/4, residues 439–563) bind RIII (residues 950–1507). An intermediate fragment of N-CoR-spanning residues 400–1040 did not interact with either fragment of ETO (Fig. 2C). Further analysis reduced these N-CoR fragments to 125–150 amino acids each (Fig. 2D). These interactions could be reproduced in a reciprocal experiment using GST fusion proteins of N-CoR (N-CoR-(1–407) or N-CoR-(950–1275)) as bait for the interaction with 35S-labeled full-length ETO (data not shown).

Deletion analysis of ETO delineated two conserved regions as being responsible for the observed protein-protein interaction with N-CoR (Fig. 3A). One region involves the NHR1 domain, and a second region involves both NHR3 and NHR4 (Fig. 3, B and C). These regions of ETO interact with RI and RIII of N-CoR, respectively. To test whether the identified interactions can also take place within mammalian cells, immunoprecipitation assays were performed using transiently transfected HEK-293 cells. A minimal RI (N-CoR-(254–407)) or RIII (N-CoR-(950–1275)) fragment of N-CoR was Myc-tagged and shown to co-precipitate with co-expressed AML1-ETO (Fig. 3D), although co-precipitation of the minimal RI fragment was significantly weaker. These results confirmed that the interaction of N-CoR RI and RIII with ETO observed in vitro resembles the interactions that can occur between these proteins in a cell.

Repressor Domain I of N-CoR Synergizes with ETO to Mediate Repression—Gal4 fusions of N-CoR RI (residues 1–407) were tested for their ability to synergize with ETO to repress transcription from a luciferase reporter. An overall 21-fold repression of the 5×Gal4-tk-luc reporter gene was observed with increasing N-CoR RI expression (see Western blot in Fig. 4A), indicating that RI can actively repress transcription (Fig. 4A). When co-expressed with ETO, the level of repression of the reporter gene was synergistically enhanced because we observed the same level of repression by RI when one-third as much expression vector was co-transfected with ETO (Fig. 4B). ETO alone had no repressive effect on the reporter in this experiment (Fig. 4B). RI also contains one of two putative Sin3 interaction domains of N-CoR (SID1) (37), and co-expression of Sin3 with this fragment resulted in the same level of repression as co-expression with ETO (Fig. 4B). Sin3 and ETO appear to complement one another, achieving the same level of repression when both proteins are co-expressed with RI (Fig. 4B). This finding suggests that there is no competition between

![Fig. 4](http://www.jbc.org/)

**Fig. 4.** N-CoR RI and ETO cooperate synergistically in repression. A, increasing amounts of the Gal4-fusion Gal4 N-CoR RI-(1–407) were transfected into CV-1 cells together with 100 ng of the 5×Gal4-tk-luc reporter gene. The level of repression increases as a function of the amount of transfected expression vector. The amount of transfected DNA reflects the amount of protein seen in a Western blot performed with the anti-Gal4 antibody. B, co-expression of ETO or Sin3A with Gal4 N-CoR RI enhances repression of the 5×Gal4-tk-luc reporter gene. When 100 ng of Gal4 N-CoR RI were transfected with 200 ng of ETO expression vector, repression was enhanced from the 5-fold observed with Gal4 N-CoR alone to 23-fold compared with the value observed with the empty vector. A similar enhancement of repression was observed with co-transfection of Sin3. When 100 ng of Sin3 and 100 ng of ETO were co-transfected with 100 ng of Gal4 N-CoR RI, the same level of repression was achieved as that observed when 200 ng of either ETO or Sin3 expression vectors were transfected, respectively. Transfection of ETO or Sin3 alone has no effect on the reporter gene. Values are given in fold repression compared with the values gained with the empty vector. The total DNA amount was adjusted with empty vector to 400 ng of DNA/transfection. Error bars represent the ± S.D. of three independent experiments. All of the values are normalized to β-galactosidase activity.
with GST N-CoR RIII and 35S-labeled ETO NHR3/4 demonstrated that each double Cys mutant diminished the binding to N-CoR RIII (Fig. 7A, right). The same pairwise NHR4 mutants were introduced into a Gal4 fusion of full-length ETO and tested for their ability to repress the 5×Gal4-tk-luc reporter gene. To avoid comparison at saturating amounts of ETO protein, the repressive activity was measured at several concentrations of transfected DNA. A Western blot against the Gal4 domain of the fusion protein was performed to show that the constructs were expressed at a similar level. The mutants (Fig. 7B) showed variable deficiency in the ability to repress the reporter gene (Fig. 7B). The Gal4 ETO-C-Ser515/535 mutant only modestly impaired repression at high amounts of transfected DNA (200 ng). By contrast, the Gal4 ETO-C-Ser526/529 and Gal4 ETO-C-Ser535/536 proteins were as defective in repression as the deletion mutant, which removed both putative zinc fingers in their entirety (Gal4 ETO-(1–515)). This indicates that, at least in this context, replacing two cysteine residues within the same putative zinc finger represents a total loss of the zinc-binding domain function.

**DISCUSSION**

ETO (MTG8) is part of a small gene family implicated in a variety of biological functions. ETO and ETO-2 (MTG16) were both identified by their association with AML1 (RUNX1) in chimeric proteins resulting from gene fusion events found in leukemia patients (14, 16, 18). Much of what is known regarding the actions of ETO in the nucleus comes from studies of these leukemic fusion proteins, the most common of which is the t(8;21) translocation gene product AML1-ETO (39–41). In this context, where nearly the entire ETO protein is fused to the DNA-binding domain of AML1, ETO appears to act as a dominant transcriptional repressor of RUNX1 gene regulation (24, 31). One mechanism by which AML1-ETO might suppress gene activation became apparent from the discovery that ETO can interact with a number of repressor proteins, notably the nuclear receptor corepressor N-CoR, the closely related co-repressor SMRT, and the co-repressor protein Sin3 (22, 24). The observation that multiple conserved regions of ETO may directly contact co-repressors and the observed co-immunoprecipitation of ETO with co-repressors and HDACs suggests that ETO forms part of a multiprotein transcriptional repression complex (31, 40, 41).

Deletion analysis of ETO suggested that at least two regions of ETO form direct interactions with a repression complex in the cell. The C-terminal NHR4 domain contains a pair of predicted zinc finger motifs, which were found to be critical to the interaction with N-CoR (Figs. 2, 3, and 7) (22–25, 36). Clearly, the putative zinc fingers alone were insufficient for this interaction, because a minimal fragment containing the zinc-binding domain (residues 490–563) failed to associate with N-CoR in vitro, in contrast to a larger fragment (residues 439–563) harboring sequences N-terminal to the putative zinc fingers themselves (Fig. 3) (23). Zinc binding appears to be essential to the ability of NHR4 to bind N-CoR, because pairwise substitution of cysteine residues in this region disrupts the interaction with N-CoR and renders ETO unable to repress transcription from a luciferase reporter (Fig. 7) (24, 41, 42). Dual Cys-to-Ser mutations at residues 515 and 535, but not other pairings, had only a modest effect on the repressive capacity of ETO (Fig. 7B), despite the observation that its direct interaction with N-CoR is significantly reduced (Fig 7A, right). This result would not be predicted from the canonical structure of zinc fingers themselves, because removal of any one of the four zinc-binding residues should disrupt the ability to bind zinc and destabilize protein structure. Given that residues 515 and 535 are putative zinc-binding residues located in each finger of the domain, the
modest reduction in repressive capacity by this double mutant might indicate a non-canonical structure for these fingers in ETO. The MYND-type zinc fingers resident in NHR4 of ETO have also been identified in other co-repressor proteins, notably human BS69, the skeletal and heart muscle specific m-Bop (mouse CD8b opposite) protein in mice, the Caenorhabditis elegans proteins BRA-1 and BRA-2, and the Myc-related cellular protein MGA (38, 43). BS69 and m-Bop have been shown to interact with a conserved binding motif, PXLXP, in cellular and oncoviral proteins, an interaction that is dependent on the MYND domain (38, 43–45). Consistent with these observations, ETO NHR3/4 binds a motif with a PPLX pattern in N-CoR (Fig. 5). The first Pro and the central Leu appear to be most important to the protein interaction (Fig. 5B). Substitution of the central Leu for Ala in the N-CoR motif diminishes the ETO/N-CoR co-repression of a luciferase reporter (Fig. 6). The Leu-to-Ala mutation introduced into this conserved binding motif in SMRT showed similar effects on the interaction between SMRT and ETO in vitro. Thus, a common binding motif for cofactors has been identified that is critical to the function of ETO as a co-repressor-binding protein.

Although regions N-terminal to NHR4 have been implicated in the interaction of ETO with co-repressor proteins (19, 25, 36), previous studies had not identified NHR1 as a participant in transcriptional repression. NHR1 harbors a motif with significant sequence similarity to Drosophila TAF$_{I110}$ and human TAF$_{I135}$ and TAF$_{I105}$. In transcriptional contexts, TAF$_{I110}$ is implicated in mediating transcriptional activation by the CCAAT-binding factor (46) and the constitutive activation domain of the CREB (47). Here, NHR1 appears to contribute to transcriptional repression by ETO (Figs. 1 and 4) and interacts with RI of N-CoR, overlapping one of the putative Sin3-binding domains. Sin3 and ETO cooperate with one another in mediating repression from a luciferase reporter driven solely by the RI domain of N-CoR (Fig. 4). Thus, in the absence of the NHR3/4 binding motif of N-CoR, ETO is still capable of mediating transcriptional repression in an N-CoR-dependent manner. This finding suggests that multiple regions of ETO can participate in a repressive complex and that it need not require the formation of a large oligomeric complex of ETO multimerized by NHR2 as has been suggested previously (25, 34, 48). Thus, the context in which repressive complexes form must be characterized to understand which portion(s) of ETO are mediating the response.

The regions of N-CoR that interact with ETO are repression domains I and III, respectively. Both of these domains harbor transferable repressive function (Figs. 4A and 6A) (9). Because RI interacts with HDAC3 and RIII interacts with HDACs 4, 5, and 7 (13), transcriptional repression by these domains is most probably mediated via the recruitment of histone. RI and RIII are also implicated in the interaction with a number of tissuespecific transcription factors, notably the pituitary-specific transcription factor, Pit-1 (49). RI is thought to interact with the myogenic differentiation antigen, MyoD (50), whereas RIII binds the B-cell lymphoma 6 protein, Bcl-6 (30). Therefore, it is

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likely that N-CoR can participate in a number of protein-protein interactions with cell type-specific factors to mediate a transcriptional response. When two interacting proteins are co-expressed with N-CoR, they could be present in the same complex as suggested in Fig. 4B for ETO and Sin3. ETO is believed to play an important role in the development of leukemia, because it is part of the leukemic fusion protein AML1-ETO. To understand the function of ETO in development and disease, it is important to decipher the molecular architecture of the co-repressor complex involving ETO, N-CoR, Sin3, and other oncoproteins involved such as Bcl-6. Deciphering which interactions with ETO can take place simultaneously and which interactions are mutually exclusive will be an important step in the analysis of the molecular targets of ETO.

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FIG. 7. Zinc binding by NHR4 of ETO is required for full repression. A, ETO contains a MYND-type double zinc finger motif. Double cysteine to serine mutations were introduced into an ETO NHR2/4 construct (amino acids 439–563) as indicated by the color code (left). The mutants show impaired binding to N-CoR RIII (950–1275) in a GST pull-down assay (right). B, repressive potential of a full-length Gal4 ETO fusion and the Cyso-to-Ser double mutants on the Gal4-tk-luc reporter gene. 100 ng of reporter gene was transfected into CV-1 cells. All of the constructs expressed to a similar degree as tested by Western blot using the anti-Gal4 antibody and extracts made from cells transfected with the highest level of DNA shown. Values are given in fold repression compared with the values gained with the empty vector. The total DNA amount was adjusted with empty vector to 400 ng of DNA/transfection. Error bars represent the ± S.D. of three independent experiments. All of the values are normalized to β-galactosidase activity. wt, wild type.
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