Fusion Protein of Retinoic Acid Receptor α with Promyelocytic Leukemia Protein or Promyelocytic Leukemia Zinc Finger Protein Recruits N-CoR-TBLR1 Corepressor Complex to Repress Transcription *in Vivo**

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Fusion proteins of retinoic acid receptor α (RARα) with promyelocytic leukemia protein (PML-RARα) or with promyelocytic leukemia zinc finger protein (PLZF-RARα) are associated with and likely responsible for the development of acute promyelocytic leukemia. These oncoproteins retain the ability to bind DNA and retinoic acid through the RARα moiety. This enables them to repress RARα target genes in the absence of retinoic acid, but the underlying mechanisms remain to be investigated. Here we use the frog oocyte system to study transcriptional regulation by PML-RARα and PLZF-RARα in the context of chromatin. We first show that the endogenous corepressor N-CoR forms a complex with TBLR1 (transducin beta-like protein 1-related protein) and that both N-CoR and TBLR1 can interact with unliganded PML-RARα and PLZF-RARα in vivo. Using chromatin immunoprecipitation, we demonstrate that both oncoproteins recruit TBLR1, as well as N-CoR, to its target promoter, leading to histone deacetylation and transcriptional repression. Furthermore, expression of a dominant negative N-CoR that contains the TBLR1-interacting domain blocks transcription repression by unliganded PML-RARα and PLZF-RARα. Thus, our studies provide in vivo evidence for targeted recruitment of N-CoR-TBLR1 complexes by PML-RARα and PLZF-RARα in transcriptional repression in the context of chromatin.

Transcriptional regulation by many kinds of transcription factors involves coactivator and corepressor complexes. The highly related corepressors N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid hormone receptors) were first identified as proteins capable of binding unliganded thyroid hormone (T_3) receptor $(TR)^1$ and retinoic acid receptor (RAR) and acting as transcription core-

pressors (1, 2). Subsequently, numerous other transcription factors have been shown to interact with N-CoR and SMRT (3). Of particular interest among them are fusion proteins implicated in causing human leukemia, including PML-RARα, PLZF-RARα, AML1-ETO, and TEL-AML1, suggesting that N-CoR and SMRT play a crucial role in leukemogenesis (4–14).

Both PML-RARα and PLZF-RARα are RARα fusion proteins that retain the ability to bind retinoic acid (RA) and RA response elements (RAREs) (15–21). This enables them to repress RAR target genes in the absence of RA and thus contribute to leukemogenesis (14). Like RAR, PML-RARα and PLZF-RARα are believed to recruit HDAC-containing corepressor complexes to the RAR targets to repress their transcription. However, direct *in vivo* evidence to support this has been lacking.

Biochemical purification of N-CoR and SMRT complexes has revealed the existence of multiple HDAC-containing complexes from HeLa cells and frog oocytes (22–30). The best characterized is the N-CoR or SMRT complex first identified in HeLa cells that contains HDAC3 and TBL1 (transducin beta-like protein 1) (23, 24). Subsequently, GPS2 (G-protein pathway suppressor 2) was also shown to be a component of this TBL1 or TBLR1 (TBL1-related protein 1) complex (26, 27).

In the present study, we investigated the possibility that this complex is utilized by PML-RARα and PLZF-RARα to repress transcription *in vivo* in the context of chromatin. For this purpose, we took advantage of the ability of the oocyte nucleus to chromatinize microinjected exogenous DNA and the lack of sufficient endogenous RAR to regulate transcription of target genes (31, 32). By expressing PML-RARα and PLZF-RARα together with their heterodimerization partner RXRα in the frog oocyte through microinjection of their mRNAs into the cytoplasm, we showed that both PML-RARα and PLZF-RARα repress RA-responsive promoter assembled into chromatin *in vivo*. To study possible involvement of N-CoR-TBL1/TBLR1 complex in this repression, we cloned the frog TBLR1 and demonstrated its association with N-CoR. By using coimmunoprecipitation (IP) and chromatin immunoprecipitation (ChIP) assays, we showed that unliganded PML-RARα and PLZF-RARα associate with both N-CoR and TBLR1 *in vivo* and recruit them to RA-responsive promoters in the context of chromatin. Furthermore, by using a dominant negative N-CoR, which contains the TBLR1-binding domain, we demonstrated a critical role of N-CoR-TBLR1 interaction in gene repression by PML-RARα and PLZF-RARα under *in vivo* chromatin conditions.

MATERIALS AND METHODS

Cloning and Vectors—The N- and C-terminal amino acid sequences of human TBL1 protein (GenBank™ accession number Y12781) were

[1] The abbreviations used are: TR, thyroid hormone receptor; RA, retinoic acid; RAR, RA receptor; RARE, RA response element; PML, promyelocytic leukemia protein; PLZF, promyelocytic leukemia zinc finger protein; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation; RT, reverse transcription; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; Ac, acetylated; MNase, micrococcal nuclease; CGID, corepressor (N-CoR) and GPS2-interacting domain; F-, FLAG-tagged; HDAC, histone deacetylase.

used for a BLAST search (Translated BLAST Search (t-blastn), www. ncbi.nlm.nih.gov/BLAST/). Five *Xenopus* expressed sequence tag clones (N-terminal: BG814471, BJ072201, and BJ042895, C-terminal: BJ033637 and BJ056849) showed sequence similarity to human TBL1. Two PCR primers (5′-CGG CGG CGG CCG ATG AGT ATA AGC AGT GAT GAG-3′ and 5′-CGG CGG ACT AGT CTA TTT TCG TAG GTC TAA TAC A-3′) were made based on these sequences and used for RT-PCR (SuperScript One-Step RT-PCR with Platinum *Taq*; Invitrogen) cloning of *Xenopus* TBL1 by using total RNA from stage 66 *Xenopus laevis* tadpoles. The PCR product was directly cloned into pCRT7-TOPO vector (Invitrogen). Three different clones (pCRT7-TBLR1) were sequenced by the DNA Sequencing Facility Center for Biosystems Research at the University of Maryland (www.umbi.umd.edu/~cab/Dna.html) and found to encode a protein (TBLR1; GenBank™ accession AY225088) more similar to human TBLR1 than TBL1.

For expression and detection in frog oocytes, a FLAG tag was added to the N terminus of human RARα (14), human PML-RARα (14), and human PLZF-RARα (10) by PCR with a primer containing the FLAG sequence. The PCR products were cloned into the T7Ts expression vector (a gift from Dr. G. J. C. Veenstra, University of Nijmegen), which is based on the pGEM-4Z vector (Promega) and contains the 5′- and 3′-untranslated regions of *X. laevis* β-globin gene flanking the multiple cloning sites.

The dominant negative N-CoR, F-DN-RD1, was made by PCR cloning of the DNA fragment corresponding to the TBL1-interacting domain (amino acids 154–304) of *Xenopus* N-CoR (33) (GenBank™ accession number AF495886). A FLAG tag and a nuclear localization signal sequence were added by PCR and cloned into T7Ts expression vector using the PCR primers 5′-AGA TCT ACC GGT GCC **ATG GAC TAC AAA GAC GAT GAC GAT AAA** (FLAG) GGA TCC **CCA AAG AAG AAG CGT AAG GTA** (nuclear localization signal) CTC GAG ATG TCT GGC CAA CCT GGA GAT-3′ and 5′-GCC GCC ACT AGT TCA ATC ATA GCG CTG ACA AAT GTT-3′. The pGL-RARE luciferase reporter vector (RARE-Luc) was a gift from Dr. Shinobu Tsuzuki (Aichi Cancer Center Research Institute, Nagoya, Japan). The backbone of this construct is pGL3-basic (Promega) and contains the DR5 sequence (5′-TCTAG**GGTTCA**CCGAA**AGTTCA**CTCGGATC-3′), the RAR/RXR binding site, followed by the TK promoter sequence inserted into the multiple cloning site of the pGL3 vector.

Antibody Preparation and Purification—Rabbit anti-*Xenopus* N-CoR serum (33) was affinity-purified using glutathione *S*-transferase-tagged *Xenopus* N-CoR N terminus fragment (amino acids 155–264). Histidine-Xpress-tagged *Xenopus* TBLR1 was obtained from *Escherichia coli* by using the pCRT7-TBLR1 construct. Rabbit anti-TBLR1 antiserum was raised against this protein and was affinity-purified using the glutathione *S*-transferase-tagged N terminus of TBLR1 (amino acids 1–211). Rabbit anti-FLAG polyclonal antibody was purchased from Affinity BioReagents (Golden, CO).

Immunoprecipitation—T7Ts-FLAG-RARα, T7Ts-FLAG-PML-RARα, T7Ts-FLAG-PLZF-RARα, and pSP64-RXR (34) were used to make mRNA with T7 or SP6 *in vitro* transcription kits (mMESSAGE mMACHINE; Ambion). The mRNA (5.75 ng/oocyte) was microinjected into the cytoplasm of 20 *X. laevis* stage VI oocytes. After incubation overnight at 18 °C, the oocytes were lysed by pipetting in IP buffer (20 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM β-glycerophosphate, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM DTT, 0.2 mM PMSF, and protease inhibitor mixture (Roche Applied Science)). After centrifugation, the supernatant was used for IP with anti-FLAG-M2-agarose beads (Sigma) or affinity-purified rabbit anti-*Xenopus* N-CoR and TBLR1 polyclonal antibodies cross-linked to protein A-agarose. Each lysate was incubated with appropriate beads for 4 h and washed three times in IP buffer. The immunoprecipitates were eluted using 100 mM glycine and immunoblotted with specific antibodies.

Transcription Assay in Xenopus Oocyte System—Stage VI oocytes from *X. laevis* were injected into the cytoplasm with different mRNAs (5.75 ng/oocyte for RAR/RXR, PML-RAR/RXR, or PLZF-RAR/RXR). Luciferase reporter plasmid (0.33 ng/oocyte) and the control vector (0.03 ng/oocyte) pRG-SV40 (Promega) were injected into the germinal vesicle (nucleus) after mRNA injection. After overnight incubation at 18 °C in the presence or absence of 10 μM of RA, the oocyte lysates were subjected to the dual luciferase assay system (Promega). Six oocytes were used for each luciferase assay. Triplicated assays were performed at the same time, and the experiments were repeated three times. The ratio of firefly luciferase activity from the RA reporter plasmid to that from the control *Renilla* luciferase plasmid was determined for each assay group, and the average from the three repeats was plotted together with the standard deviation.

Chromatin Immunoprecipitation—The ChIP assay for recruitment of

corepressor complexes and histone acetylation were essentially as described (35). F-PML-RAR mRNA and RARE-Luc reporter DNA were injected into 20 oocytes. After overnight incubation, the oocytes were treated with 1% formaldehyde for 10 min to cross-link the DNA to histones and/or other associated proteins. After washing the oocytes with the incubation medium, the oocytes were incubated in 1 ml of 100 mM Tris-HCl, pH 9.4, 10 mM DTT for 15 min at 30 °C to stop the cross-linking reaction. The oocytes were lysed by pipetting in 400 μl of homogenization buffer (20 mM Tris-HCl, pH 7.6, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 1 mM DTT, and protease inhibitor mixture tablet (Roche Applied Science)). The mixture was then sonicated to fragment the plasmid minichromosome (5 s for five times on ice with Branson Sonifire 450, output 2, duty, cycle 30). It is then diluted with 400 μl of ChIP I buffer (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM DTT, and 0.4 mM PMSF) and centrifuged at 15,000 rpm (20,000 g) in an Eppendorf centrifuge for 10 min. The supernatant was mixed with 60 μl of salmon sperm DNA/protein A-agarose beads (Upstate Biotechnology Inc.). After incubation at 4 °C for 2 h, the sample was centrifuged again, and 200 μl of this precleared supernatant were used for IP with an indicated antibody. For FLAG ChIP, anti-FLAG-M2-agarose beads were used. Affinity-purified TBLR1 or N-CoR polyclonal antibody was used for TBLR1 or N-CoR ChIP. For acetylated histone H3 (Ac-H3) and H4 (Ac-sH4), anti-Ac-H3 and anti-Ac-H4 antibodies (Upstate Biotechnology Inc.) were used. Salmon sperm DNA/Protein A-agarose (Upstate Biotechnology Inc.) was used for the ChIP of TBLR1, N-CoR, Ac-H3, and Ac-H4. After overnight incubation of the antibody/beads with the precleared supernatant, the mixture was centrifuged at 400 × g for 5 min, and the beads were washed sequentially for 15 min each using the following buffer: ChIP I, ChIP II (0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 50 mM HEPES, pH 7.5, 500 mM NaCl, 1 mM DTT, and 0.4 mM PMSF), ChIP III (0.25 M LiCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0, 1 mM DTT, and 0.4 mM PMSF), and Tris-EDTA buffer (10 mM Tris/1 mM EDTA, pH 8) with 1 mM DTT and 0.4 mM PMSF. The beads were then incubated in 200 μl of elution buffer (0.5% SDS, 0.1 M NaHCO₃) with 5 μg of proteinase K at 65 °C for overnight to reverse the DNA-protein cross-links.

The eluted DNA from the beads was purified with the PCR purification kit (Qiagen). The DNA was eluted into 40 μl of Buffer EB, and 4 μl was used for each PCR reaction: 95 °C for 3 min for 1 cycle and 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 20 s for 25 cycles. Amplified DNA was loaded onto a 2% agarose gel and visualized by ethidium bromide staining after electrophoresis. PCR primers for the ChIP assay were as follows: forward 5′-ATT TTC ATT ACA TCT GTG TGT TG-3′ and reverse 5′-CAA ACC CTA ACC ACC GCT TA-3′. These primers amplify the region containing the RARE to produce fragments of 263 bp. The PCR products were quantified with BioMax 1D (Kodak, New Haven, CT).

Micrococcal Nuclease Assay—MNase assay of chromatin structure was performed as described previously (36, 37). Briefly, 0.5 ng/oocyte of RARE-Luc reporter DNA was injected into the germinal vesicle of 30 oocytes. After overnight incubation, the injected oocytes were lysed by pipetting in 300 μl of homogenization buffer (10 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 5 mM MgCl₂, and 1 mM DTT). Fifty μl of the lysate was used digestion with MNase (0, 0.5, 1, 5, or 20 units) for 20 min at room temperature. The reaction was stopped with 50 μl of 2× TNESK buffer (20 mM Tris-HCl, 200 mM NaCl, 2 mM EDTA, and 2% SDS). The mixture was treated with 10 μg of RNase A for 30 min at 37 °C, followed by treatment with 75 μg of proteinase K for 2 h at 55 °C. After phenol/chloroform extraction, DNA was purified by isopropanol precipitation and dissolved in 20 μl of water. After adding DNA loading dye, one half of each sample was electrophoresed on a 1.5% agarose gel followed by Southern blotting. For the detection, ³²P-labeled RARE-TK DNA fragment derived from RARE-Luc reporter vector was used.

RESULTS

Xenopus TBLR1 Protein Sequence Is Highly Homologous to Human TBLR1 and TBL1—Biochemical purification from several laboratories has revealed the existence of N-CoR and SMRT complexes containing TBL1 or TBLR1 in mammalian tissue culture cells (23, 24, 26, 27). As a first step toward studying the role of TBL1 or TBLR1 complexes in gene repression by unliganded nuclear receptors in the context of chromatin, we searched for the presence of such complexes in frog oocytes. We queried the *Xenopus* expressed sequence tag data base with N and C terminus amino acid sequences of human
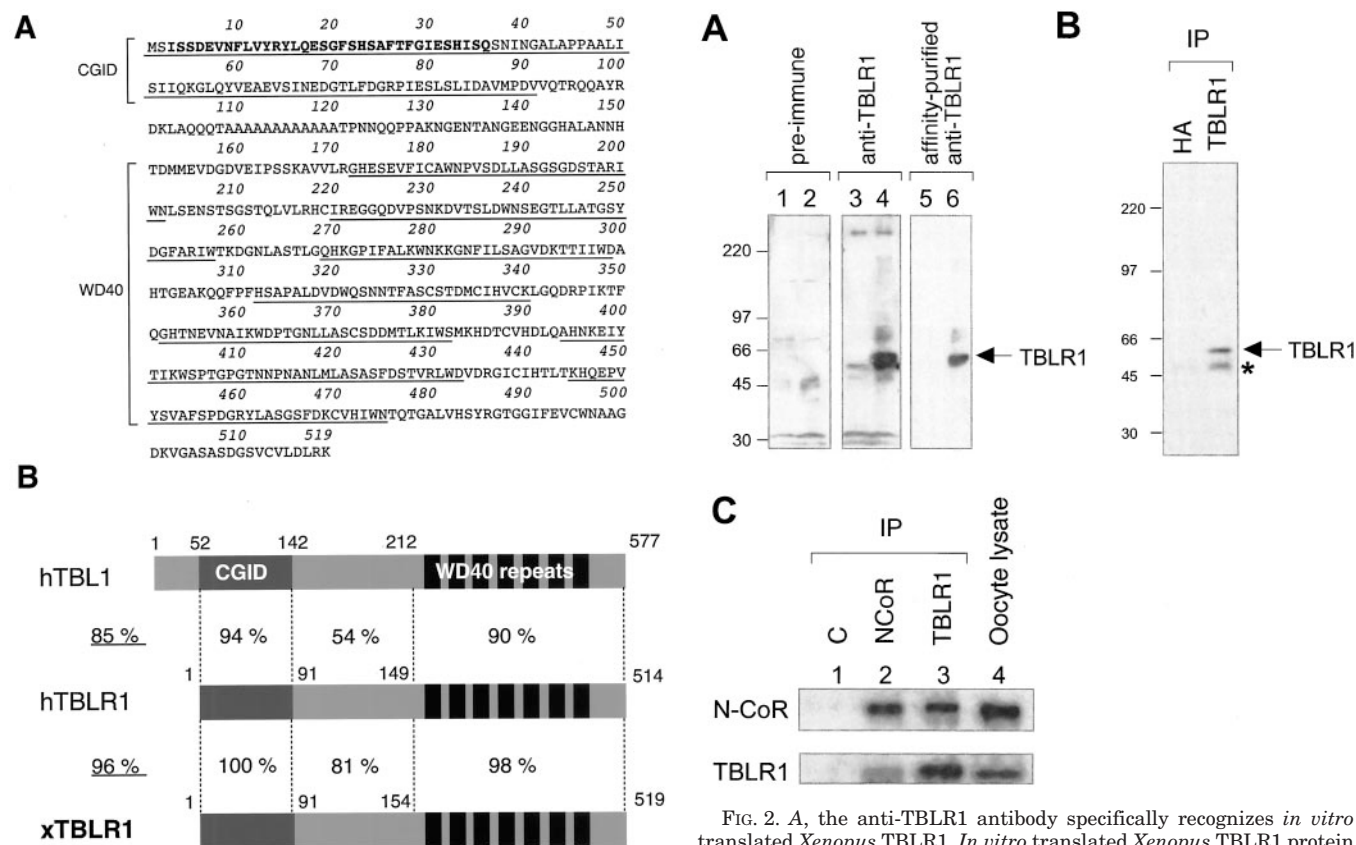
FIG. 1. *A*, amino acid sequences of *Xenopus* TBLR1 (xTBLR1) protein. CGID (26) and WD40 repeats are indicated. Seven repeats of WD40 sequences are *underlined*. LisH (Lissencephaly type-1-like homology motif) consensus sequences in CGID are indicated with *bold letters*. The cDNA sequence was deposited in GenBank™ (accession number AY225088). *B*, sequence comparison among of xTBLR1, human TBL1 (hTBL1; GenBank™ accession number Y12781), and TBLR1 (hTBLR1; GenBank™ accession number AF314544). Percentage identities for different domains between hTBLR1 and hTBL1 or xTBLR1 are shown in between the proteins. The xTBLR1 shares 96% (*underlined*) overall identity with hTBLR1, higher than the 85% (*underlined*) identity between hTBL1 and hTBL1. In particular, the CGID domain is completely identical, and the WD40 repeat domain is 98% identical between xTBLR1 and hTBLR1.

TBL1 and obtained several expressed sequence tag sequences similar to the N or C terminus of human TBL1. Two specific primers were designed to amplify the entire coding region of the gene by using RT-PCR on RNA isolated from *X. laevis* froglets. A single cDNA band (~1.6 kb) was amplified (data not shown) and cloned. Three independent clones were sequenced and found to be identical, encoding a protein of 519 amino acids (Fig. 1*A*).

This cloned *Xenopus* protein was found to be more similar to human TBLR1 than human TBL1 (Fig. 1*B*). This protein, *Xenopus* TBLR1, contains seven WD40 repeats and the corepressor (N-CoR) and GPS2-interacting domain (CGID) at the N terminus (Fig. 1). The CGID sequence is totally conserved between *Xenopus* TBLR1 and human TBLR1 and 94% identical to that of human TBL1 (Fig. 1*B*). Similarly, the WD40 repeat domain is 98% identical between *Xenopus* TBLR1 and human TBLR1, whereas only 90% identical between the two human proteins. Overall, *Xenopus* TBLR1 shares 96% identity with human TBLR1 at the amino acid sequence level (Fig. 1*B*) and 84% identity at the DNA sequence level (data not shown). Such high degrees of conservation suggest similar biochemical and molecular functions.

Endogenous TBLR1 Interacts with N-CoR in Xenopus Oocytes—To investigate whether TBLR1-N-CoR complexes exist
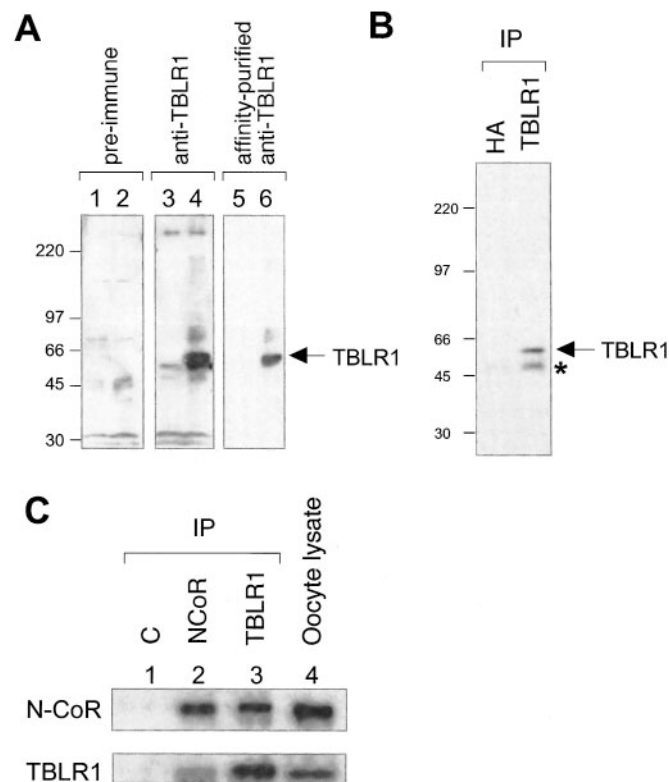

FIG. 2. *A*, the anti-TBLR1 antibody specifically recognizes *in vitro* translated *Xenopus* TBLR1. *In vitro* translated *Xenopus* TBLR1 protein (*lanes 2*, *4*, and *6*) or *in vitro* translated sample without TBLR1 construct (*lanes 1*, *3*, and *5*) were subjected to Western blotting using preimmune rabbit serum, anti-TBLR1 serum, or affinity-purified TBLR1 antibody. *B*, affinity-purified anti-TBLR1 antibody specifically immunoprecipitates the endogenous TBLR1 in oocytes. Anti-hemagglutinin or TBLR1 antibodies were used for immunoprecipitation of *Xenopus* oocyte lysate and subsequent Western blotting used affinity-purified TBLR1 antibody. The *asterisk* indicates IgG heavy chain. *C*, endogenous N-CoR and TBLR1 form a complex in *Xenopus* oocytes. Twenty-stage VI oocytes were used for each IP followed by immunoblotting on 25% of the eluted sample from the IP with the indicated antibodies. *Lane 4* shows the amount of N-CoR or TBLR1 in the lysate of 1 oocyte. Note that affinity-purified rabbit anti-N-CoR (*lane 2*) and anti-TBLR1 (*lane 3*) antibodies were able to IP both endogenous N-CoR and TBLR1 in the oocyte. Rabbit anti-hemagglutinin antibody failed to IP either protein (control, *lane 1*). By comparing the signals in *lane 1* with those in *lanes 2* and *3*, it is estimated that the N-CoR and TBLR1 antibodies immunoprecipitated roughly 10% of N-CoR and 40% TBLR1, respectively.

in *Xenopus* oocytes, we generated a polyclonal antibody against *Xenopus* TBLR1. This antibody specifically recognized *Xenopus* TBLR1 translated *in vitro* (Fig. 2*A*) and could immunoprecipitate the endogenous *Xenopus* TBLR1 in the frog oocyte (Fig. 2*B*). It failed to detect any protein of the size expected for the *Xenopus* homolog of mammalian TBL1 (data not shown), suggesting either that a putative *Xenopus* TBL1 is not expressed in the oocyte or that the antibody does not cross-react with a *Xenopus* TBL1. To investigate the association of TBLR1 and N-CoR, we immunoprecipitated these proteins from oocyte extract with affinity-purified anti-TBLR1 (Fig. 2*C*, *lane 3*) or anti-*Xenopus* N-CoR (Fig. 2*C*, *lane 2*) antibody. Western blot analysis showed that both antibodies were able to bring down both proteins (Fig. 2*C*), indicating that endogenous TBLR1 and N-CoR interact with each other in *Xenopus* oocytes.

Transcriptional Repression by Unliganded PML-RARα and PLZF-RARα Correlates with Interactions between Receptors and TBLR1 in Vivo—To study the possible involvement of TBLR1 in gene regulation by PML-RARα and PLZF-RARα *in vivo*, we made use of the frog oocyte system that we have

established to study transcriptional regulation by nuclear hormone receptors in the context of chromatin (31, 34). For this purpose, we microinjected *in vitro* transcribed mRNA encoding FLAG-tagged PML-RARα (F-PML-RAR) or PLZF-RARα (F-PLZF-RAR) together with RXR mRNA into the cytoplasm of oocytes to allow synthesis of the proteins. For comparison, we carried out the studies on RARα injecting mRNA for FLAG-tagged RARα with RXR mRNA into another batch of oocytes. A few hours later, we microinjected into the germinal vesicle (nucleus) two reporter plasmids, one containing an RA-dependent promoter driving the expression of the firefly luciferase and the other containing a control promoter driving the expression of *Renilla* luciferase. After an overnight incubation with or without RA, the oocytes were lysed for the luciferase assay. As expected, unliganded RAR/RXR repressed the target gene, whereas RA-bound RAR/RXR activated it (Fig. 3A, *lanes 1–3*), as shown earlier (31). Similarly, both F-PML-RAR and F-PLZF-RAR were able to repress the RA-dependent promoter in a chromatin template *in vivo* and RA treatment reversed this repression (Fig. 3A, *lanes 4–9*). It is worth noting that although both RAR and PML-RAR were able to activate the promoter above its basal level in the presence of RA, RA merely reversed the repression caused by PLZF-RAR, consistent with earlier studies showing that PLZF-RAR is more resistant to RA treatment (10–12).

To investigate whether the repression by RAR and the RAR fusion proteins involves N-CoR-TBLR1 complex, similarly treated oocytes were lysed and subjected to IP using anti-FLAG affinity agarose beads. Western blot analysis showed that endogenous N-CoR and TBLR1 associated with F-PML-RAR and F-PLZF-RAR as well as RAR in the absence of RA (Fig. 3B). Two other corepressors, Sin3 or Rpd3/HDAC1/2, that were shown to be able to interact with N-CoR *in vivo* and in tissue culture cells (38–41) failed to associate with F-PML-RAR, F-PLZF-RAR, or FLAG-tagged RAR (Fig. 3B and not shown). RA treatment released both N-CoR and TBLR1 from the receptors (Fig. 3B).

PML-RARα Recruits Endogenous N-CoR and TBLR1 to the Target Promoter in Chromatin—To study the molecular mechanism of receptor-mediated repression at the RARE-containing promoter, we carried out ChIP assay to determine the proteins that were bound to the promoter region in chromatin. First, to ensure that the reporter DNA injected into the oocyte nucleus was chromatinized, we carried out MNase digestion assay on plasmid minichromosome isolated from oocytes after overnight incubation. Southern blotting with a probe made of an RARE-containing promoter fragment from the RARE-Luc reporter DNA showed the presence of a normal nucleosomal array on the plasmid minichromosome as indicated by the formation of a nucleosomal DNA ladder upon limited MNase digestion (Fig. 4).

For ChIP assay, the oocytes were injected with mRNAs encoding F-PML-RAR and RXR as well as the reporter plasmid and were incubated overnight in the presence or absence of RA. They were then treated with formaldehyde to cross-link the plasmid reporter DNA with the associated proteins in the plasmid minichromosome *in vivo*. After sonication to break the DNA to about 500 bp in size, the DNA-protein cross-links were immunoprecipitated with the FLAG antibody against F-PML-RAR expressed in the oocyte. The precipitated DNA was then analyzed for the presence of the promoter region containing RARE. The result clearly demonstrated the binding of F-PML-RAR to the promoter regions independent of RA treatment (Fig. 5, *A* and *B*, compare *lanes 2* and *3* with *lane 1* for the FLAG ChIP). To determine whether N-CoR and TBLR1 were recruited by F-PML-RAR to the promoter, the same cross-
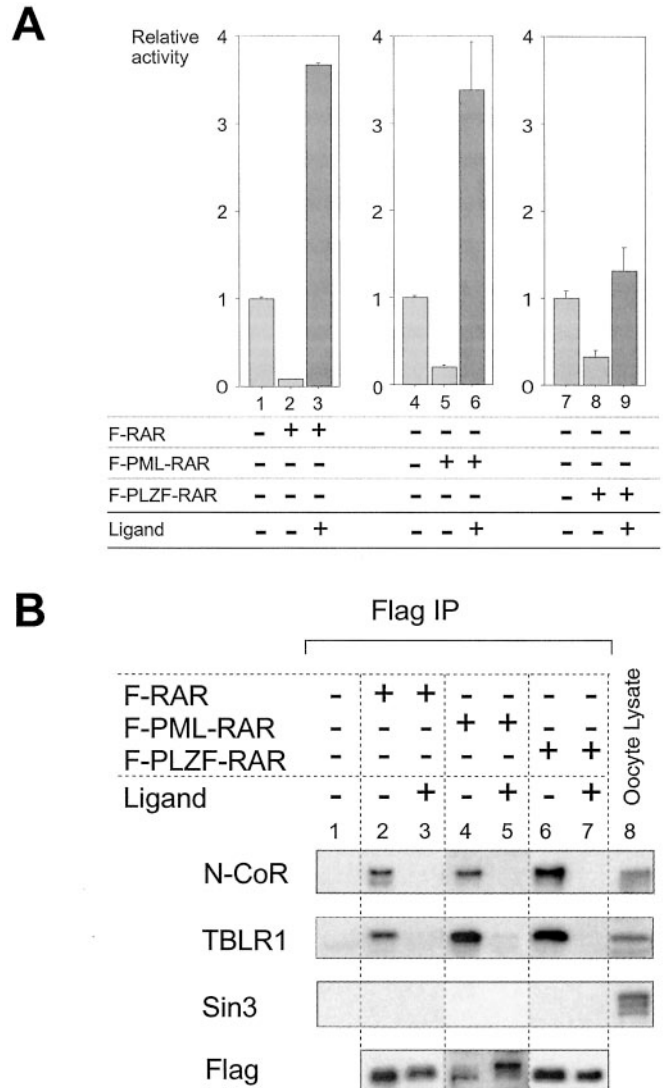
FIG. 3. **Unliganded nuclear receptors repress transcription *in vivo* on chromatinized templates and associate with N-CoR and TBLR1 but not Sin3 in the frog oocyte.** *A*, PML-RARα, PLZF-RARα, and RARα regulate transcription in a ligand-dependent manner in the oocyte system. The mRNA (5.75 ng/oocyte) for FLAG-tagged RARα, PML-RARα, or PLZF-RARα was injected into the cytoplasm of 20 oocytes together with RXR mRNA (5.75 ng/oocyte) as indicated. The firefly luciferase reporter vector (0.33 ng/oocyte) (RARE-Luc) was then injected into the nucleus together with the control *Renilla* luciferase plasmid (0.03 ng/oocyte). The oocytes were treated with or without 10 μM RA as indicated. After overnight incubation, the oocytes were harvested for the dual luciferase assay. Six oocytes were used for each luciferase assay. Triplicated assays were performed at the same time, and the experiments were repeated three times. The ratio of firefly luciferase activity from RARE-Luc to that from the control *Renilla* luciferase plasmid was determined for each assay group, and the average from the three repeats was plotted together with the standard deviation. *B*, PML-RARα, PLZF-RARα, and RARα interact with endogenous N-CoR and TBLR1 but not Sin3 in the oocyte. The oocytes were injected and incubated as in *A*. IP using anti-FLAG affinity beads was performed on 20 oocytes and 25% of the eluted sample was subjected to SDS-PAGE-Western blot analysis. Rabbit anti-N-CoR, TBLR1, or Sin3 antibody was used for the detection of each endogenous protein, and anti-FLAG antibody was used to determine the efficiency of IP. Note that all receptors interacted with N-CoR and TBLR1 in the absence of ligand, and ligand induced their dissociation from N-CoR and TBLR1. No interaction with Sin3 was detectable for any receptors under our experimental conditions. The IP on uninjected oocytes is shown in *lane 1* as a negative control. Oocyte lysate (*lane 8*) was used as a positive control. The slower mobility of the F-PML-RAR band as detected by the FLAG antibody (*lane 5*) might be due to ligand-induced, post-translational modification of the protein, such as sumoylation (58).
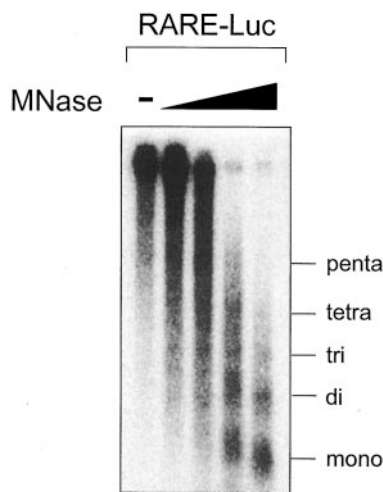
FIG. 4. **The reporter DNA is assembled into chromatin in the frog oocyte.** RARE-Luc (0.5 ng) was injected into the germinal vesicle of frog oocytes, and after incubation for 14 h, the oocytes were lysed and subjected to MNase digestion with different amounts of MNase (0, 0.5, 1, 5, and 20 units). As a probe for Southern blotting, RARE-containing promoter fragment from the RARE-Luc reporter DNA was used. Note the formation of DNA fragments corresponding to mono-, di-, tri-, tetra, and penta-nucleosomes upon limited MNase digestion, indicative of a normal nucleosomal array on the plasmid minichromosome.

linked DNA-protein complexes were subjected to immunoprecipitation with antibodies against endogenous N-CoR and TBLR1. PCR analyses of the precipitated DNA showed that in the absence of RA, both F-PML-RAR recruited both N-CoR and TBLR1 to the promoter, and this recruitment was abolished upon RA treatment (Fig. 5, compare *lanes 2* and *3* with *lane 1*). Because N-CoR-TBLR1 complex contains HDAC3 (23, 24, 26, 27), we then analyzed the acetylation levels at the promoter. Consistent with the recruitment of the complex, the acetylation levels of both histones H3 and H4 were reduced concurrently with the recruitment of N-CoR and TBLR1 (Fig. 5, compare *lane 2* with *lane 1*). Upon ligand addition, the histone acetylation levels were restored (Fig. 5, compare *lane 3* with *lane 2*). Although the lack of antibody against endogenous HDAC3 (the *Xenopus* HDAC3 has not been cloned) prevented direct analysis of the recruitment of this HDAC to the promoter, these data strongly suggest that N-CoR-TBLR1-HDAC corepressor complexes are recruited by unliganded PML-RARα, leading to histone deacetylation and gene repression.

The TBLR1-interacting Domain of N-CoR (DN-RD1) Disrupts Interactions between Endogenous TBLR1 and N-CoR and Inhibits Gene Repression by Unliganded Nuclear Receptors—To test the role of TBLR1-N-CoR complex in transcription regulation by unliganded nuclear receptors, we investigated whether interfering with the association between N-CoR and TBLR1 *in vivo* alters gene regulation by nuclear receptors. We reasoned that overexpression of the TBLR1-interacting region of N-CoR may disrupt endogenous N-CoR-TBLR1 complexes by functioning as a competitive inhibitor. Thus, we prepared a construct encoding an N-terminal FLAG-tagged *Xenopus* N-CoR fragment corresponding to the TBL1-interacting domain of human N-CoR (Fig. 6*A*) (24, 26).

We tested whether this fragment (F-DN-RD1) could interact with endogenous TBLR1 in oocytes. The mRNA encoding F-DN-RD1 was injected into oocytes, and after overnight incubation, the oocyte lysate was subjected to IP using anti-FLAG affinity beads. Western blot analysis showed that endogenous TBLR1 interacts with F-DN-RD1 (Fig. 6*B*).

To determine whether F-DN-RD1 can interfere with endogenous TBLR1-N-CoR interaction, we microinjected increasing
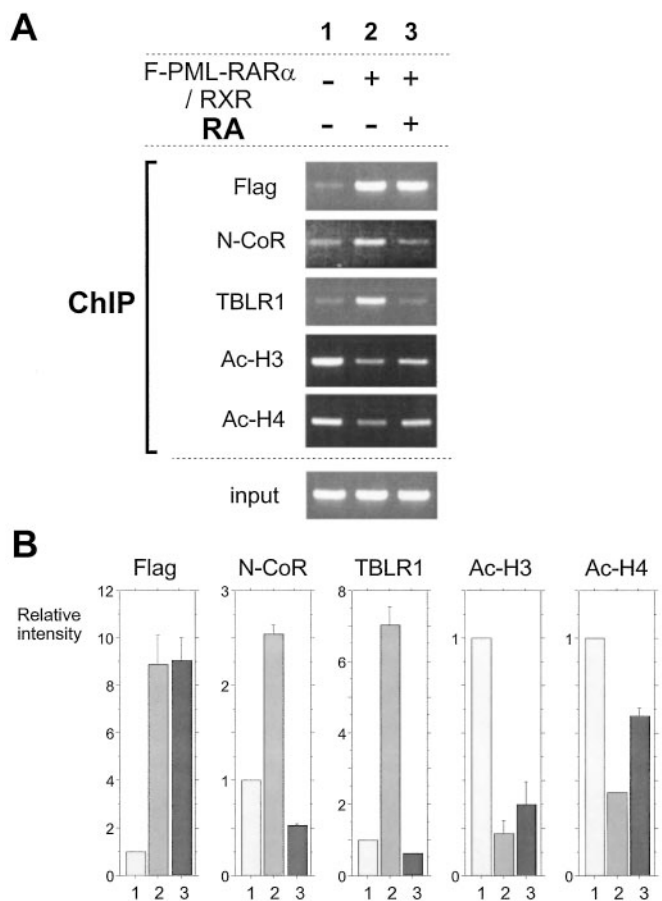

FIG. 5. **PML-RAR recruits N-CoR and TBLR1 to target promoters and induce histone deacetylation in the absence of RA.** *A*, agarose gel analysis of the ChIP assay products. Oocytes were injected and incubated as in Fig. 3. They were then processed for ChIP assay with antibodies against FLAG (for F-PML-RAR), N-CoR, TBLR1, acetylated histone H3 (Ac-H3), and Ac-H4. The presence of the RARE region of the promoter in the immunoprecipitated DNA was determined by PCR using primer pairs flanking the RARE region. The PCR products were analyzed on a 2% agarose gel containing ethidium bromide. The DNA prior to IP was amplified as the *input* control to show equal amounts of DNA in all samples. *B*, quantification of the data shown in *A*. The intensities of PCR bands were quantified from two independent ChIP assays with the signal in *lane 1* set to 1. Note that both PML-RAR binds to the promoter constitutively and recruits N-CoR and TBL1 in the absence of RA, leading to deacetylation of both H3 and H4 at the promoter. The addition of RA dissociates the corepressors and restores histone acetylation at the promoter.

amounts of the mRNA encoding F-DN-RD1 into the oocytes. After an overnight incubation, the oocytes were lysed and subjected to IP with anti-TBLR1 antibody to immunoprecipitate endogenous TBLR1 followed by Western blot analysis with anti-TBLR1, FLAG, or N-CoR antibodies. As expected, endogenous TBLR1 was associated with N-CoR (Fig. 6*C*, *lane 2*). With increasing amounts of F-DN-RD1 mRNA, increasing amounts of F-DN-RD1 was found to be immunoprecipitated by the anti-TBLR1 antibody (Fig. 6*C*, *lanes 2–5*), indicating that F-DN-RD1 associated with endogenous TBLR1. Conversely, the association of endogenous N-CoR with TBLR1 was reduced by about 3-fold with the highest level of F-DN-RD1 expressed in the oocytes (Fig. 6*C*, *lane 5*). Thus, F-DN-RD1 protein was able to disrupt endogenous TBLR1-N-CoR complexes.

We next studied the consequence of the disruption of the TBLR1-N-CoR interaction on transcriptional repression by RAR/RXR, PML-RAR/RXR, or PLZF-RAR/RXR (Fig. 7). We microinjected increasing amounts of F-DN-RD1 mRNA together with mRNAs for RAR/RXR, PML-RAR/RXR, or PLZF-
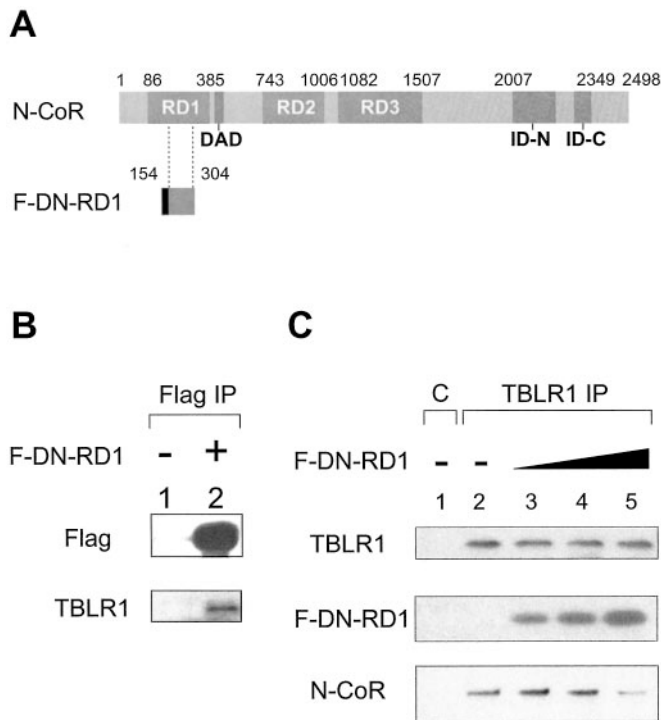
FIG. 6. **A dominant negative form of *Xenopus* N-CoR competes for association with TBLR1 *in vivo*.** *A*, schematic representation of full-length *Xenopus* N-CoR (*upper*) protein and its dominant negative construct encoding FLAG-tagged TBLR1 binding domain (*lower*; F-DN-RD1), which is located in the repression domain 1 (*RD1*) (26). The deacetylase activating domain (*DAD*), two nuclear hormone receptor interacting domains (*ID-N* and *ID-C*), and amino acid numbers are indicated. *B*, endogenous TBLR1 interacts with F-DN-RD1. The oocytes were injected without (*lane 1*) or with (*lane 2*) mRNA for F-DN-RD1. After overnight incubation, IP assay using FLAG affinity beads was performed followed by Western blot analysis with anti-FLAG or anti-TBLR1 antibody. *C*, F-DN-RD1 displaces endogenous N-CoR from TBLR1. F-DN-RD1 mRNA was injected at 1.2, 4.6, and 23 ng/oocyte (*lanes 3–5*, respectively) into 20 oocytes and incubated overnight. The oocyte extracts were subjected to IP using anti-TBLR1 antibody (*lanes 2–5*). As a control, protein A beads without antibody were used for IP in *lane 1*. Five oocyte equivalents of IP eluates were subjected to Western blot analysis with anti-TBLR1 and -N-CoR antibodies. Note that the anti-N-CoR antibody recognizes both endogenous N-CoR and F-DN-RD1. With increasing F-DN-RD1 expression, more F-DN-RD1 was immunoprecipitated with TBLR1, and less endogenous full-length N-CoR came down (*lane 5*).

RAR/RXR, followed by nuclear injection of reporter DNA. After an overnight incubation, the oocytes were harvested for luciferase assay. Again, unliganded RAR/RXR, PML-RAR/RXR, or PLZF-RAR/RXR repressed their respective target promoter. Overexpression of F-DN-RD1 was able to reverse the repression by all these receptors (Fig. 7), indicating that TBLR1-N-CoR interaction is critical for repression by these proteins.

DISCUSSION

The transforming proteins for acute promyelocytic leukemia are fusion proteins of nuclear hormone receptor RARα. They retain the DNA and hormone binding properties of the receptor, thereby allowing them to regulate the expression of RAR target genes. Many nuclear hormone receptors, including RAR and TR, have dual functions, repressing target genes in the absence of ligand and activating them when ligand is present. *In vitro* and tissue culture cell studies have shown that nuclear hormone receptors interact with a number of cofactor complexes (42–49). In the absence of ligand, they bind corepressors, such as SMRT and N-CoR, which form multimeric complexes containing histone deacetylases, whereas in the presence of ligand, they interact with coactivator complexes, such as SRC-1, CBP/p300, or DRIP/TRAP complexes, many but not all of which have histone acetyltransferase activity (22–24, 40–42, 45, 47, 50–54). Despite the enormous knowledge on these complexes, relatively little is known about their utilization by different transcription factors *in vivo*.

Transcriptional repression by unliganded nuclear receptors has received a lot of attention in recent years because of increasing evidence implicating unliganded receptors in developmental and pathological processes (48, 55–57). Despite this, little is known about how unliganded nuclear receptors, especially oncoproteins PML-RARα and PLZF-RARα, regulate transcription *in vivo*. In this paper, by using an *in vivo* system that allows us to study transcription mechanisms in the context of chromatin, we demonstrated that *in vivo* PML-RARα and PZLF-RARα 1) bind to RARE in chromatin constitutively; 2) interact with TBLR1, likely through N-CoR, in the absence of RA; 3) recruit both N-CoR and TBLR1 to RARE, leading to histone deacetylation; and 4) require N-CoR-TBLR1 interaction for transcriptional repression. Our data thus support a role for N-CoR-TBLR1-HDAC or a related complex in mediating transcriptional repression and leukemogenesis by PML-RARα and PZLF-RARα.

The highly related large corepressors N-CoR and SMRT were first identified as proteins that bind to unliganded TR and RAR (1, 2). Subsequently, both were found to interact with the corepressor Sin3, which in turn binds to Rpd3 (HDAC1/2) (38–41). This led to the suggestion that N-CoR-Sin3-Rpd3-containing corepressor complexes mediate the repression by unliganded nuclear hormone receptors. On the other hand, no evidence has been reported for *in vivo* association of unliganded nuclear receptors with such an N-CoR-Sin3-Rpd3 complex. Our results here show that PML-RARα, PLZF-RARα, and RARα fail to associate with Sin3-Rpd3 under our immunoprecipitation conditions.

Biochemical studies and complex purification have revealed the existence of multiple N-CoR/SMRT-HDAC complexes, including an N-CoR-HDAC complex containing Sin3 and Rpd3 in the frog oocyte (22). The best characterized N-CoR/SMRT-HDAC complexes are those containing TBL1 or TBLR1 and HDAC3 isolated from HeLa cells (23, 24, 26, 27). Our data here show the existence of similar N-CoR complexes in the frog oocyte. Furthermore, our IP data indicate that unliganded RARα and its fusion proteins PML-RARα and PLZF-RARα interact *in vivo* with N-CoR-TBLR1 complexes but not Sin3 or Rpd3 and that the receptor-bound protein complexes have HDAC activity (data not shown). In addition, our ChIP assay provides *in vivo* evidence that TBLR1 is recruited by PML-RARα to target genes in the absence of ligand and that the addition of ligand abolishes this recruitment. Concurrent with this recruitment, acetylation levels at target promoters are reduced, in agreement with the presence of HDAC activity in N-CoR-TBLR1 complexes (23, 24, 26, 27).

The ability of the TBLR1 interaction domain of N-CoR to function as a dominant negative *in vivo* supports the importance of N-CoR-TBLR1 complexes in transcriptional repression by PML-RARα and PLZF-RARα. Although this N-CoR fragment may also interact with other proteins *in vivo*, such as putative *Xenopus* TBL1 and GPS2, it clearly inhibits the interaction of the endogenous wild type N-CoR and TBLR1 in a dose-dependent manner. Although the inability to use knockout or knockdown approaches, such as RNA interference in the frog oocyte, this data together with the ability of PML-RARα or PLZF-RARα to interact with TBLR1 (likely through N-CoR) and recruit it to the promoter argues that the disruption of TBLR1-N-CoR interaction is responsible for inhibiting the repression by PML-RARα and PLZF-RARα. On the other hand, it
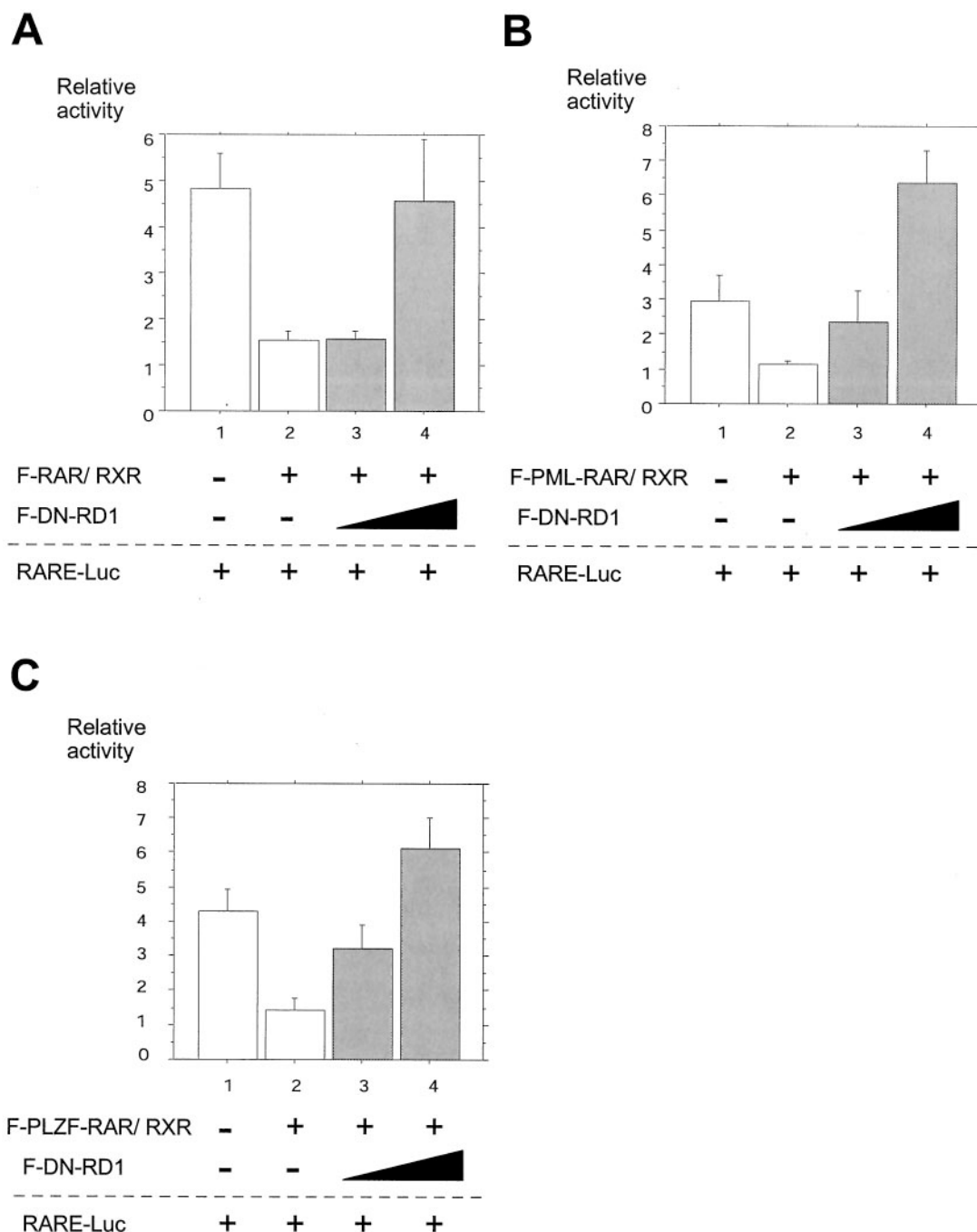
A

B

C

FIG. 7. **F-DN-RD1 reverses the transcription repression by unliganded RARα (*A*), PML-RARα (*B*), and PLZF-RARα (*C*).** The oocytes were injected without or with 4.6 or 23 ng/oocyte mRNA for F-DN-RD1 together with RXR and indicated receptor mRNAs followed by the injection of the luciferase reporter DNA as in Fig. 3. After overnight incubation, the oocytes were harvested for luciferase assay to determine the transcription from the RAR-dependent promoter as in Fig. 3. Note that unliganded receptors repress reporter expression and with increasing amounts of F-DN-RD1, the reporter activity increased, showing the anti-repression effect of F-DN-RD1.

is worth pointing out that because of a lack of appropriate reagents, we cannot rule out that endogenous TBL1-N-CoR interaction and/or TBL1-SMRT or TBLR1-SMRT interactions are also disrupted by the dominant negative because it should interact with both TBL1 and TBLR1. Regardless, our data support the model that PML-RARα, PLZF-RARα, and RARα recruit N-CoR-TBLR1 complexes or related complexes to deacetylate local chromatin at promoters to mediate gene repression in the absence of RA.

PML-RARα and PLZF-RARα are believed to play a critical role in the development of acute promyelocytic leukemia (15–21). Similar to TR and RAR as discussed above, the ability of PML-RARα and PLZF-RARα to interact with N-CoR and

SMRT has led to the suggestion that these oncoproteins interact with N-CoR/SMRT-Sin3-HDAC1(Rpd3) complex (4, 10–12), although no direct *in vivo* evidence is available. Furthermore, it has been reported that PLZF-RARα has two N-CoR interaction domains, the PLZF-derived N-terminal domain and the RAR-derived C-terminal domain, and that the N-terminal interaction domain of PLZF-RARα does not respond to RA, thus enabling PLZF-RARα to be more resistant to RA than PML-RARα (4, 10–13). Our results indicate that both PML-RARα and PLZF-RARα interact with N-CoR-TBLR1 complexes but not N-CoR-Sin3-Rpd3 complexes. Furthermore, we show that this interaction with N-CoR and TBLR1 by both PML-RARα and PLZF-RARα can be disrupted by RA *in vivo*. This disruption is

consistent with the ability of RA to abolish the repression by unliganded PML-RARα and PLZF-RARα. On the other hand, under our experimental conditions, RA failed to activate the promoter in case of PLZF-RARα. This suggests that PLZF-RARα may have altered ability to recruit coactivators compared with PML-RARα, thus leading to the increased resistance of PLZF-RARα to RA compared with PML-RARα. Regardless, the ability of RA or TBLR1-interacting dominant negative N-CoR to reverse the repression by both PML-RARα and PLZF-RARα points to the significance of N-CoR-TBLR1 or related complexes in gene regulation by these oncoproteins. Given the effectiveness of RA in leukemia treatment and our findings here, it is quite likely that N-CoR-TBLR1 complexes or highly related complexes play a critical role in human leukemogenesis, and targeting N-CoR-TBLR1 interaction may provide another promising avenue for prevention and/or treatment of acute promyelocytic leukemia.

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