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# CoREST: A functional corepressor required for regulation of neural-specific gene expression

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**ABSTRACT** **Several genes encoding proteins critical to the neuronal phenotype, such as the brain type II sodium channel gene, are expressed to high levels only in neurons. This cell specificity is due, in part, to long-term repression in nonneural cells mediated by the repressor protein REST/NRSF (RE1 silencing transcription factor/neural-restrictive silencing factor). We show here that CoREST, a newly identified human protein, functions as a corepressor for REST. A single zinc finger motif in REST is required for CoREST interaction. Mutations of the motif that disrupt binding also abrogate repression. When fused to a Gal4 DNA-binding domain, CoREST functions as a repressor. CoREST is present in cell lines that express REST, and the proteins are found in the same immunocomplex. CoREST contains two SANT (SW13/ADA2/NCoR/TFIIIB B) domains, a structural feature of the nuclear receptor and silencing mediator for retinoid and thyroid human receptors (SMRT)-extended corepressors that mediate inducible repression by steroid hormone receptors. Together, REST and CoREST mediate repression of the type II sodium channel promoter in nonneural cells, and the REST/CoREST complex may mediate long-term repression essential to maintenance of cell identity.**

A large number of genes encoding neuronal phenotypic traits, including ion channels, neurotransmitters, synaptic proteins, and cell-adhesion molecules, are expressed only in neurons. One mechanism important in establishing and maintaining this neural specificity involves the DNA-binding protein REST/NRSF (RE1 silencing transcription factor/neural-restrictive silencing factor) (1–4), which serves to block expression of its target genes in nonneural tissues. Such maintained gene repression is in contrast to the more dynamic repression mechanism that regulates inducible gene expression in response to steroid hormone receptors, one of the best-studied mammalian repressor mechanisms (for review, see ref. 5).

One REST target gene essential for neuronal physiology is that encoding the brain type II voltage-dependent sodium channel. This ion channel is required for the propagation of fast electrical signals in neurons, in the form of neuronal impulses, and is not expressed in nonneural tissues. As is true for other REST target genes, there is a reciprocal relationship between expression of the type II sodium channel gene and expression of REST. Additionally, when a REST expression plasmid is cotransfected into neuronal cells along with a type II sodium channel reporter, the expression of the reporter gene is reduced dramatically (1). This result indicates either that REST alone is sufficient to repress its target genes or that REST accessory factors are present in neuronal cells despite the absence of REST.

Two distinct repressor domains have been identified and characterized in REST (6, 7). These domains are located in the amino and carboxyl termini of the protein. Both domains are required for full repression in the context of the intact molecule, but each domain is sufficient to repress type II sodium channel reporter genes when expressed as a Gal4 fusion protein (6). The C-terminal repressor domain contains a $C_2H_2$ class zinc finger beginning approximately 40 aa upstream of the stop codon. Deleting this domain, or introducing a point mutation critical to the zinc finger motif, abolishes repressor activity (6). Because zinc finger motifs often mediate protein–protein interactions, we proposed that REST might function in conjunction with other nuclear factors or corepressors.

In this study, we find that repression of the type II sodium channel promoter by REST requires a newly identified protein, CoREST, which fulfills the criteria for a bona fide corepressor. CoREST is a repressor; mutations that disrupt CoREST's binding to REST also interfere with REST repression. Endogenous REST and CoREST proteins form a complex in cells that do not express the type II sodium channel gene.

## MATERIALS AND METHODS

**Two-Hybrid Screening.** The REST cDNA used for bait (LexA-C-REST) contains amino acids 525-1097 of REST. G. Hannon (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) provided the HeLa cell cDNA library. Library screens were carried out, as described previously (8, 9), by using HIS3 and LacZ reporters and the L40 strain provided by R. Sternglanz (State University of New York at Stony Brook). Specificity of the interaction was examined by a mating assay between the positive L40 transformants and an AMR-70 strain expressing REST-related proteins [LexA-N-REST, amino acids 1–525; LexA-C-RESTM1, amino acids 525-1097 (Cys-1062 → Arg); LexA-C3-REST, amino acids 1013–1097] and several LexA fusion proteins not related to REST. All cDNAs were characterized by sequence analysis.

**Library Screening.** A λ Zap HeLa cDNA library (Stratagene) was screened with a cDNA probe representing a 5′

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Abbreviations: REST, RE1 silencing transcription factor; NRSF, neural-restrictive silencing factor; SMRT, silencing mediator for retinoid and thyroid hormone receptor; SMRTe, SMRT-extended; NCoR, nuclear receptor corepressor; SANT, SW13/ADA2/NCoR/TFIIIB; GST, glutathione *S*-transferase; DBD, DNA-binding domain; CAT, chloramphenicol acetyltransferase.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF155595).

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fragment of KIAA0071 (10). Positive clones were excised from the phagemid according to the manufacturer's instructions (Stratagene) and characterized by chain-termination sequence analysis (Amersham Pharmacia) by using an ABI 300 automated sequencer (Applied Biosystems). One clone extended the published sequence of KIAA0071 by 101 aa and contains 227 nt of 5′ untranslated region. This cDNA (termed CoR5b) was joined to KIAA0071 to generate full-length CoREST (described below).

**Plasmid Constructions.** A CoREST expression vector (pcDNACoREST) was constructed by inserting an *Eco*RI-*Bst*XI fragment from Cor5b and a *Bst*XI-*Xba*I fragment from KIAA0071 into pcDNA1.1amp (Invitrogen). Cloning a *Bsa*BI/*Eco*RI-blunted fragment of CoREST cDNA into pcDNA3.1A (Invitrogen) linearized with *Eco*RV created an epitope-tagged version of CoREST (CoREST myc). Cloning CoREST cDNA into vector pSG424 (6) created Gal4CoREST. The glutathione *S*-transferase (GST) CoREST vector used in the pulldown assays contains amino acids 109–293 of CoREST. The CoREST sequences were placed in-frame with GST in the vector pGEX-3X (Amersham Pharmacia). PCR and subsequent cloning into the vector pCGN (provided by J. Trimmer, State University of New York at Stony Brook) created an epitope-tagged version of the zinc finger domain of the C terminus of REST (HA-C3, amino acids 1009–1097). LexA-N-REST (amino acids 1–525) was created by a three-way ligation among pBTM116 cut with *Bam*HI and *Sal*I-blunt, a *Bam*HI-*Cla*I fragment of pCBY REST2, and a *Cla*I-*Hin*cII fragment of REST. LexA-C-REST (amino acids 525-1097) was created by cloning a *Hin*dII-*Pst*I fragment of NH7 (1) into pBTM116 cut with *Bam*HI-blunt and *Pst*I. LexA-C-RESTM1 (amino acids 525-1097, Cys1062 → Arg) was created by a three-way ligation of an *Eae*I-*Pst*I fragment from C3M1 (6) plus a *Hin*dII-*Pst*I fragment of NH7 (1) into pBTM116 cut with *Bam*HI and *Pst*I. LexA-C3-REST (amino acids 1013–1097) was generated by cloning a PCR fragment containing amino acids 1013–1097 into pBTM116 cut with *Bam*HI and *Pst*I. The REST cDNAs lacking the N-terminal REST domain (RESTΔN; REEX 8) lacking the C-terminal zinc finger domain (RESTΔC; REEX9) or containing a point mutation in the zinc finger motif (RESTM1; Cys-1062 → Arg) have been described previously (6). Gal4C3

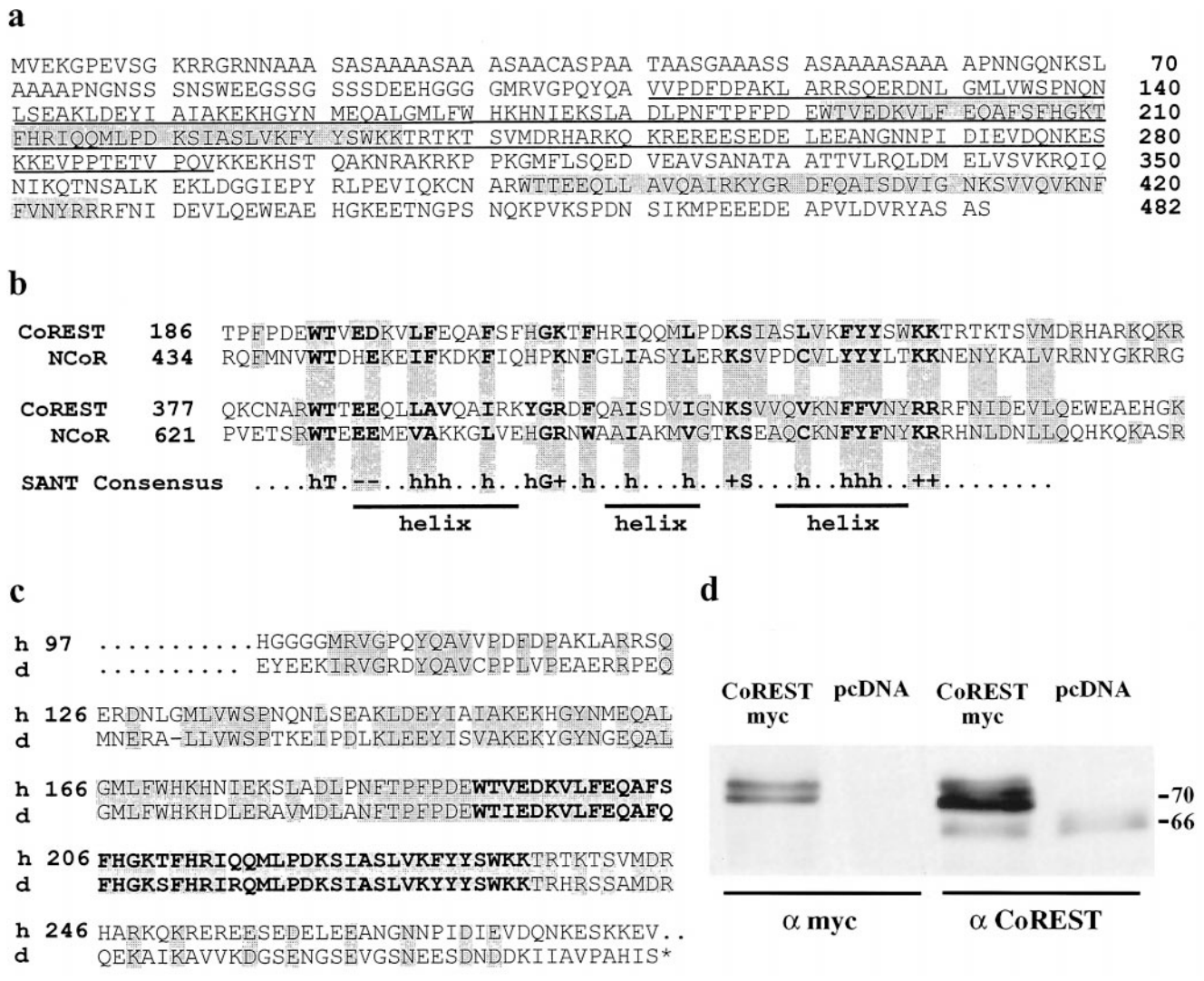


FIG. 1. Characterization of CoREST. (*a*) Predicted amino acid sequence of human CoREST (hCoREST) (accession no. AF155595). The underlined amino acids represent the protein fragment isolated in the yeast two-hybrid screen. The two SANT domains (15) are shaded. (*b*) Alignment of the two repeated SANT domains in CoREST and NCoR. For comparison, the SANT consensus and predicted structure are shown below. h, hydrophobic residues; + and −, positively and negatively charged residues. Amino acids conforming to the SANT consensus are in boldface. Additional homologies between CoREST and NCoR are shaded. (*c*) Alignment of hCoREST (h) with a similar *Drosophila* protein (d) (accession no. AA392295). Conserved amino acids are shaded. Boldface type indicates the SANT domain. (*d*) Western blot of nuclear extracts from HEK293 cells transfected with pcDNA or with a myc-tagged CoREST cDNA. The probes were a polyclonal CoREST antibody (αCoREST) and a monoclonal myc antibody (αmyc). The positions of migration of endogenous CoREST (66 kDa) and myc-CoREST (70 kDa) are indicated.

and the type II promoter chloramphenicol acetyltransferase (CAT) reporter genes, pSDK7 and UAS type II, were described previously (1, 6).

**GST-Pulldown Assay.** Radiolabeled REST proteins ($^{35}$S) were made in TNT-coupled transcription/translation reactions (Promega). The REST protein was incubated with 15 $\mu$g of GST or GSTCoREST (amino acids 109–293) in binding buffer (11) for 1.5 hr at 4°C, and then 80 $\mu$l of glutathione-Sepharose beads (Pharmacia) preblocked with 5% BSA was added. The mixture was incubated for 1 hr at 4°C. Beads were washed six times with binding buffer. Bound proteins were eluted by boiling the beads with sample buffer, fractionated by SDS/PAGE, and visualized by autoradiography.

**Antibodies.** Rabbits were injected with GSTCoREST (amino acids 109–293) by Pocono Rabbit Farms (Canadensis, PA). The polyclonal antibody $\alpha$CoREST was affinity-purified by following standard techniques (12). The monoclonal $\alpha$-myc and $\alpha$-hemagglutinin (HA) antibodies and the polyclonal $\alpha$-Gal4 DNA-binding domain (DBD) antibody were purchased from Santa Cruz Biotechnology.

**Immunoprecipitation and Western Blot Analyses.** Antibodies (5 $\mu$g) were incubated (1 hr, 4°C) with 1 mg of protein extracted from L6 skeletal muscle cells by lysing cells in PBS containing 0.1% Nonidet P-40/0.2 mM PMSF/2 $\mu$g/ml $\alpha$-antitrypsin/2 $\mu$g/ml aprotinin/2 $\mu$g/ml leupeptin/1 mM DTT/1 mM $Na_3VO_4$. The mixture was incubated for an additional hour with 30 $\mu$l of protein G-agarose (GIBCO). Immunoprecipitates were washed two times with washing buffer (PBS/350 mM NaCl containing 0.1% Nonidet P-40) and two times with PBS, and then were eluted in sample buffer. The immunoprecipitated proteins were analyzed by Western blotting by using rabbit polyclonal REST antibody (1) and detected by using chemiluminescence (ECL; Amersham Pharmacia). Preparation of nuclear and cytoplasmic extracts was as described previously (13).

**Cell Culture and Transient Transfection.** PC12 and L6 cells were grown as described (6). HEK293 cells were maintained in DMEM supplemented with 10% BCS. The HEK293 and PC12 cells were transfected by using FuGENE 6 (Roche) or electroporated, respectively. Control experiments were carried out by using molar equivalent amount of empty vector. The total amount of transfected DNA was kept constant by adding pBluescript SR (Stratagene). CAT assays were performed 48 hr after transfection as described (6).

## RESULTS

To identify proteins that might mediate repression through the C-terminal zinc finger motif of REST, a fragment corresponding to the C-terminal half of REST (amino acids 525–1097) was fused to the DBD of LexA and used in a yeast two-hybrid genetic screen with a HeLa cell–Gal4 activation-domain cDNA library. A screen of $7.6 \times 10^6$ clones yielded two identical cDNAs encoding a 183-aa fragment, and this fragment did not interact with several other, unrelated LexA fusion proteins (data not shown).

A search of the GenBank database revealed that the isolated cDNA was identical to a cDNA coding for a protein of unknown function isolated from a human myelocarcinoma cell line (KIAA0071; accession no. D31888; ref. 10). A full-length cDNA was isolated from a HeLa library by using KIAA0071 as probe, and the deduced ORF was found to encode a 482-aa protein (Fig. 1*a*, accession no. AF155595). The putative initiator methionine conforms to a Kozak consensus sequence (14) and is preceded by an in-frame stop codon. This protein was designated CoREST.

The predicted CoREST primary structure contains two SANT (SW13/ADA2/NCoR/TFIIIB) domains (15) separated by 191 aa (Fig. 1*b*). That a similar module exists in the nuclear receptor corepressor (NCoR)/silencing mediator for retinoid and thyroid human receptors (SMRT)-extended (SMRTe) corepressor (16, 17) and in the transcription factor

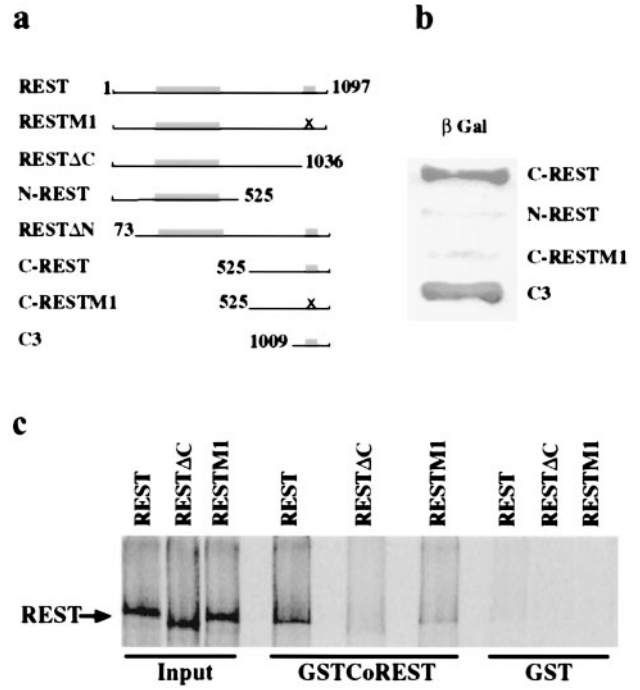


FIG. 2. CoREST interacts with the C-terminal repressor domain of REST. (*a*) The family of REST cDNAs used in *in vitro* and *in vivo* experiments with CoREST. The larger solid rectangle depicts the eight zinc finger motifs in the DBD; the smaller rectangle depicts the single zinc finger in the C terminus. REST (amino acids 1–1097) represents full-length protein. (*b*) Yeast two-hybrid interactions between pGAD-CoREST (amino acids 109–293) and four different REST-LexA fusion proteins. (*c*) REST/CoREST interactions in GST pull-down assay. Indicated REST constructs were transcribed and translated *in vitro* to yield $^{35}$S-labeled products and were incubated with immobilized GST or GSTCoREST (amino acids 109–293). Input (3% of total protein) and proteins bound to GST were analyzed by SDS/PAGE.

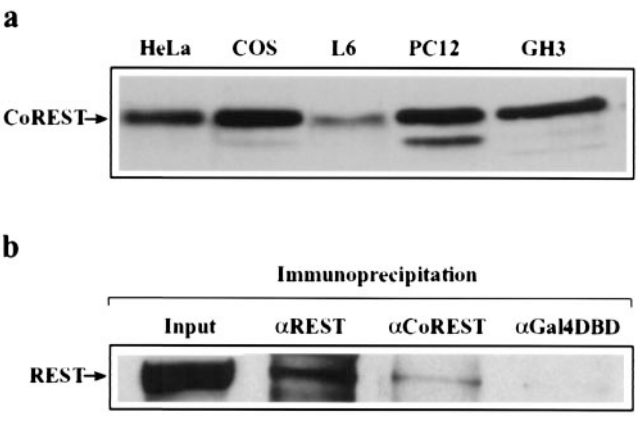


FIG. 3. (*a*) CoREST is expressed in neuronal and nonneuronal cells. Western blot of nuclear extracts (50 $\mu$g) prepared from neuronal (PC12, GH3) and nonneuronal (HeLa, COS-1, L6) cell lines. The blot was probed with $\alpha$CoREST antibody. Endogenous CoREST protein migrates at 66 kDa. (*b*) Demonstration of REST/CoREST interaction *in vivo* by coimmunoprecipitation analysis. Whole-cell lysates prepared from L6 skeletal muscle cells were immunoprecipitated with the indicated antibodies and then subjected to Western blotting. The membrane was probed with $\alpha$REST antibody (1). The arrow depicts migration of REST protein. Input corresponds to 50 $\mu$g of extract. $\alpha$CoREST and $\alpha$Gal4DBD refer to antibodies against CoREST and against the DBD of Gal4 protein, respectively.

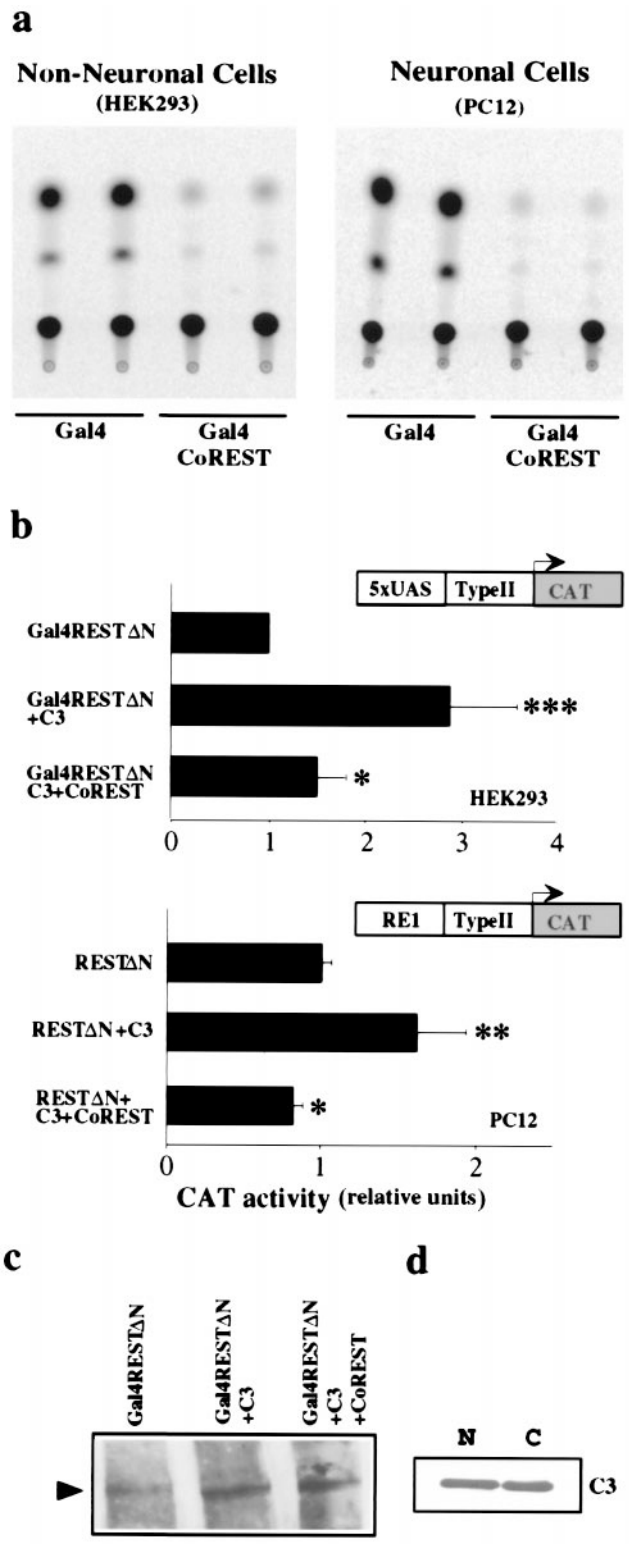


FIG. 4. CoREST exhibits repressor activity when fused to a Gal4-DBD and mediates repressor activity of REST. (*a*) Gal4-DBD (Gal4; amino acids 1–147) or Gal4CoREST expression vectors were transfected into HEK293 and PC12 cells, with a UAS type II CAT reporter gene (molar ratio of 1:1). (*b Upper*) Gal4REST lacking the N-terminal repressor domain (Gal4RESTΔN) was transfected into HEK293 cells along with a UAS type II CAT reporter gene. The role of CoREST was tested by addition of a cDNA coding for a REST competitor peptide containing the CoREST-binding site (Gal4RESTΔN + C3). Specificity of derepression was tested by over-expressing CoREST (Gal4RESTΔN + C3 + CoREST). (*Lower*) REST lacking the N-terminal repressor domain (RESTΔN) was transfected into PC12 cells along with an RE1 type II reporter gene. The role of CoREST was tested by addition of a cDNA coding for a REST competitor peptide containing the CoREST-binding site (RESTΔN + C3). Specificity of derepression was tested by overexpressing CoREST (RESTΔN + C3 + CoREST). CAT activity was monitored by TLC and autoradiography. Statistics were performed by using the nonparametric Mann–Whitney *U* test. *, $P < 0.056$ for RESTΔN + C3 +CoREST compared with RESTΔN + C3 and Gal4RESTΔN + C3 + CoREST compared with Gal4RESTΔN + C3; **, $P < 0.0028$ for RESTΔN + C3 compared with RESTΔN; ***, $P < 0.0008$ for Gal4RESTΔN + C3 compared with Gal4RESTΔN. (*c*) Western blot of nuclear extracts of HEK293 cells transfected as in *b*. The probe was a monoclonal αGal4 antibody. (*d*) Western blot from nuclear (N) and cytoplasmic (C) extracts of HEK293 cells transfected with an HA-tagged C3. The probe was a monoclonal αHA antibody.

Adf1 (18) suggests that there is a conserved functional role for this arrangement of SANT domains. Interestingly, a *Drosophila*-expressed sequence tag (accession no. AA392295) that predicts an ORF with homology to the N-terminal portion of CoREST (dCoREST) was identified by a GenBank search. Sequence analysis of the cDNA and alignment of the predicted sequences indicates that the dCoREST and hCoREST predicted proteins are 84% identical over a span of 114 aa (Fig. 1*c*). Included in this region is a SANT domain that is nearly identical in the two proteins.

Western blot analysis, using an antibody raised against a GSTCoREST fusion protein, determined that endogenous CoREST expressed in cell lines migrates in SDS/PAGE with an apparent molecular mass of 66 kDa (Fig. 1*d*). Transfection of a myc-tagged CoREST migrates at about 70 kDa (Fig. 1*d*). Both the endogenous and the transfected CoREST migrate as a doublet, suggesting posttranslational modification. Western blotting also showed that CoREST was present in a wide variety of neural (PC12, GH3) and nonneural (HeLa, L6 skeletal muscle, COS) cell types (Fig. 3*a*). This result is in good agreement with previous Northern blot analysis, using the clone KIAA0071 as a probe, which indicated that CoREST mRNA was expressed ubiquitously in adult human tissues (unpublished data and ref. 10).

The specificity of the interaction between REST and CoREST was examined in several ways. In yeast two-hybrid assays (Fig. 2*b*) CoREST (amino acids 109–293) was found to interact strongly with the C-terminal half of REST (C-REST) (Fig. 2*a*), as well as with a smaller C-terminal REST fragment (C3) containing the zinc finger motif known to mediate repressor activity. Furthermore, a point mutation in the zinc finger motif (C-RESTM1) that abolished repression (6) also abolished the REST–CoREST interaction. CoREST did not interact with the N-terminal part of REST (N-REST) containing the initiator methionine and the eight zinc fingers that constitute the DBD.

To extend these results, a direct interaction between REST and CoREST was demonstrated by using a GST pull-down assay (Fig. 2*c*). Full-length REST (amino acids 1–1097) transcribed and translated *in vitro* was incubated with GSTCoREST or GST alone. REST associated specifically with GSTCoREST. There was no interaction between CoREST and RESTΔC (Fig. 2*a*), which lacks the C-terminal zinc finger. A point mutation in this motif (RESTM1) decreased the binding to GSTCoREST.

Cell types that express CoREST were identified by Western blotting (Fig. 3*a*). In contrast to REST, which is expressed only in nonneuronal cell lines, CoREST was expressed in all cell lines tested. Because L6 skeletal muscle cells express the highest levels of REST (data not shown), these cells were used to test for *in vivo* interactions between endogenous REST and CoREST by using αCoREST and control antibodies. Western blotting revealed the presence of REST epitopes in the αCoREST and not in control immunoprecipitates (Fig. 3*b*).

If CoREST is a bona fide corepressor, in addition to binding to REST, CoREST might exhibit repressor activity. To test this idea, CoREST was fused to the DBD of Gal4 (Gal4CoREST) and cotransfected into PC12 and HEK293 cells along with a UAS type II sodium channel CAT reporter (6). This reporter is driven by the type II promoter but is missing the RE1 sequences. Transfection with the Gal4 DBD alone had no effect on reporter expression (Fig. 4*a*). In contrast, transfection of Gal4CoREST repressed UAS type II reporter activity very effectively in HEK293 cells (between 15- and 20-fold repression; Fig. 4*a*). Furthermore, CoREST also repressed reporter activity in neuronal (PC12) cells (Fig. 4*a*), which lack REST.

To provide further evidence that CoREST mediates repression by REST, a competition assay was performed. In this assay, RESTΔN (Fig. 2*a*) was cotransfected with a cDNA coding for a small fragment of REST containing the CoREST-binding site (C3; Fig. 2*a*). The functional consequence of the competition, if endogenous CoREST indeed is mediating repression, should be an increase of the reporter activity (derepression). This result was observed using both nonneuronal (HEK293) (Fig. 4*b Upper*) and neuronal (PC12) cells (Fig. 4*b Lower*). The effect of the competitor protein could be reversed by overexpression of CoREST (Fig. 4*b*). As a control, the DBD of REST (p73; ref. 6) was transfected along with Gal4RESTΔN and C3 in HEK293 cells. Under these conditions, the activity of the reporter gene was similar to that observed with the competitor (C3) alone, showing that the effect of CoREST was specific (data not shown). Western blots showed that the expression of C3 or C3 plus CoREST in HEK293 cells did not decrease the expression of Gal4RESTΔN (Fig. 4*c*) and that C3 is present in both nuclear (N) and cytoplasmic (C) extracts of HEK293-transfected cells (Fig. 4*d*).

## DISCUSSION

We have identified a factor, CoREST, that is part of the repressor mechanism leading to neural-specific expression of a large set of genes. CoREST and REST are present in a complex in nonneuronal cells, and CoREST exhibits repressor activity. Previous studies have shown that a C-terminal zinc finger domain in REST mediates repression (6). We find that the site of CoREST binding to REST maps to this domain. Further, the same mutation that disrupts repressor activity of REST by destroying the zinc finger motif (6) also blocks binding of CoREST. Thus, CoREST is an essential component of the repression mechanism used to block expression of type II sodium channel reporter genes in nonneuronal cell types.

The predicted primary structure of CoREST contains a repeated 50-aa motif termed a SANT domain (15). A SANT domain is similar to the DBDs of myb oncoproteins. CoREST contains two SANT domains separated by 191 aa, and the same general arrangement also is found in the NCoR/SMRTe corepressors for steroid hormone receptors (16, 17). In addition, between the SANT domains, CoREST and NCoR/SMRTe contain a highly charged region. The function of the SANT domains in corepressors is not known, but in Adf1, a transcriptional activator that contains a similar dual-SANT module, one SANT domain binds to DNA and the other is a site of protein–protein interaction (18). CoREST is not known to bind to any specific DNA sequence, but the first SANT domain contributes to the binding to REST (unpublished data). It is possible that CoREST helps stabilize binding of REST to DNA via its SANT module, or, alternatively, CoREST may be involved in mediating other functions that require these domains. The identification of a *Drosophila* protein that is homologous to the amino terminus of CoREST raises the possibility that some of the functions of CoREST, in addition to those mediated by the SANT domains, are conserved in invertebrates.

The experiments showing that Gal4CoREST is a repressor in neuronal PC12 cells (Fig. 4*a*) suggest that the function of REST largely is to recruit the repression complex to the target DNA in nonneuronal cells. These data, together with the finding that CoREST is present in complexes with endogenous REST in nonneuronal cells (Fig. 3*b*), indicate that CoREST mediates REST repression through the REST C-terminal domain. The competition experiments, whereby a REST fragment that interferes with CoREST binding to REST also derepresses a type II reporter, further support this hypothesis. Western and Northern blot analyses (data not shown and ref. 10) indicate that, unlike REST, CoREST is expressed ubiquitously *in vivo*. In particular, CoREST is present in brain, where REST, in general, is expressed at very low levels. The more widespread pattern of expression of CoREST in adult animals suggests that, in addition to serving as a corepressor for REST, CoREST might subserve a similar function for other DNA-binding proteins as well.

The details of the mechanism by which REST represses its target genes are not known. It is possible that CoREST interacts and interferes with components of the basal transcriptional apparatus. Alternatively, CoREST could function as a repressor by recruiting, either directly or indirectly, histone deacetylase activity (19–23). The corepressors NCoR/SMRTe apparently repress through both mechanisms (19, 23, 24). In this regard, it will be interesting to determine whether CoREST is independent of the NCoR/SMRTe pathway.

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