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Regulation of Neuronal Traits by a Novel Transcriptional Complex

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Summary

The transcriptional repressor, REST, helps restrict neuronal traits to neurons by blocking their expression in nonneuronal cells. To examine the repercussions of REST expression in neurons, we generated a neuronal cell line that expresses REST conditionally. REST expression inhibited differentiation by nerve growth factor, suppressing both sodium current and neurite growth. A novel corepressor complex, CoREST/HDAC2, was shown to be required for REST repression. In the presence of REST, the CoREST/HDAC2 complex occupied the native Nav1.2 sodium channel gene in chromatin. In neuronal cells that lack REST and express sodium channels, the corepressor complex was not present on the gene. Collectively, these studies define a novel HDAC complex that is recruited by the C-terminal repressor domain of REST to actively repress genes essential to the neuronal phenotype.

Introduction

Inhibitory pathways play important roles throughout neuronal development, from neural induction to the acquisition of the terminally differentiated phenotype (for reviews see Anderson and Jan, 1997; Edlund and Jessell, 1999). Moreover, a large number of genes encoding neuronal phenotypic traits, such as ion channels, neurotransmitter receptors, and synaptic vesicle proteins, are actively repressed in nonneuronal cells. Amongst these genes are the Nav1.2 sodium channel (Kraner et al., 1992; Maue et al., 1990), SCG10 (Mori et al., 1990), synapsin I (Howland et al., 1991; Li et al., 1993; Schoch et al., 1996), and subunits of the muscarinic acetylcholine receptor (Mieda et al., 1997; Wood et al., 1996), the nicotinic acetylcholine receptor (Bessis et al., 1997; Bessis et al., 1995), and the glutamate receptor (Myers et al., 1998). A commonality among these genes is the presence of a conserved 23 bp repressor element, known as RE1 or NRSE (repressor element 1/neuron restrictive silencer element) (Chong et al., 1995; Schoenherr et al., 1996). Removal of the RE1 sequences from transgenes containing a portion of the regulatory region results in ectopic expression in nonneural tissues and, occasionally, in neurons (Kallunki et al., 1998; Wuenschell et al., 1990). Based upon these data, the RE1 binding silencer protein, REST (also called NRSF) (Chong et al., 1995; Schoenherr and Anderson, 1995), has been hypothesized to serve as a critical modulator of expression of neuronal traits. Consistent with this idea, REST is required for normal mouse development, and transient expression of a dominant negative form of REST in nonneural tissues results in precocious and ectopic expression of some of its target genes (Chen et al., 1998). Conversely, virally mediated overexpression of REST in chick spinal cord commissural neurons results in axonal path-finding defects (Paquette et al., 2000). These observations suggest that downregulation of REST during neurogenesis is required for proper development and for the acquisition of the terminally differentiated phenotype. The molecular mechanism by which REST exerts such regulatory effects, however, is not known.

Large repressor complexes (2 Mda) (Li et al., 2000) have been purified and dissected functionally in yeast and mammals. In these complexes, corepressors have been identified that can potentially modify chromatin structure through posttranslational modifications of histones (for reviews, see Ayer, 1999; Knoepfler and Eisenman, 1999; Pazin and Kadonaga, 1997; Tyler and Kadonaga, 1999; Vignali et al., 2000; Wolffe and Guschin, 2000). These corepressor complexes include the mSin3A/B complex, which contains the class I HDACs 1 and 2; the NuRD complex, which contains an ATPase (Mi2) as well as HDACs; and the N-CoR complex, which contains HDAC3 (Jones et al., 2001; Li et al., 2000; Wen et al., 2000). Intriguingly, REST contains two distinct repressor domains, one located in the N terminus and the other located in the C terminus of the protein (Tapia-Ramirez et al., 1997; Thiel et al., 1998). The corepressor mSin3A/B interacts directly with the N terminus of REST and is required for repression through this domain (Grimes et al., 2000; Huang et al., 1999; Naruse et al., 1999; Roopra et al., 2000). A corepressor, termed CoREST, was first identified by its interaction with the C-terminal repressor domain of REST (Andres et al., 1999). Point mutations in the C-terminal zinc finger motif of REST disrupt the interaction between REST and CoREST and abrogate REST repressor activity (Andres

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et al., 1999). The mechanism of REST repression of genes, mediated by CoREST, has not been determined.

In this study, we use a conditional expression system to explore the dynamics and role of the REST/CoREST repressor complex in control of the neuronal phenotype. We show that in the presence of CoREST, REST interferes with the differentiation program induced by nerve growth factor (NGF). Both the induction of sodium currents, due to expression of the endogenous Nav1.2 sodium channel gene, and the extension of neurites in PC12 cells are significantly reduced by expression of REST. CoREST is shown to exist in tight association with HDAC1/2, and single-cell antibody microinjection analysis indicates that REST, CoREST, and HDAC2 are all required for repression of the Nav1.2 sodium channel promoter in nonneuronal cells. Chromatin immunoprecipitation assays reveal that the REST complex occupies both the native Nav1.2 and SCG10 genes in nonneuronal cells but not in neuronal cells. These studies elucidate the components of an active repression mechanism that is important in restricting neuronal traits to neurons.

Results

REST Suppresses Acquisition of Neuronal Traits in PC12 Cells Treated with Nerve Growth Factor

One of the best-characterized REST target genes encodes the rat Nav1.2 voltage-dependent sodium channel (Nav1.2). This channel is expressed at high levels only in neurons where it is responsible for propagation of neuronal impulses. Analysis of the effects of persistent expression of REST on Nav1.2 sodium channel excitability in vivo is complicated by the difficulties in associating neuronal sodium currents with a specific sodium channel type. To circumvent this problem, we exploited PC12 cells where induction of sodium currents in response to NGF is due to increased expression of the Nav1.2 sodium channel gene (Mandel et al., 1988). To examine the effects of REST, PC12 clonal lines expressing REST cDNA under control of an inducible promoter were generated.

A PC12 cell line containing the tetracycline-VP16 activator fusion protein was characterized for its ability to respond normally to a 48 hr exposure to NGF. This line, which we refer to herein as the parental PC12 *tet* line, like other PC12 lines, does not express REST (Figure 1A). It responds normally to a 48 hr treatment with NGF, exhibiting an approximately 2.5-fold increase in Nav1.2 sodium channel mRNA and resulting in a 4-fold increase in inward sodium current (Figures 1D and 1E). The parental line also extends neurites similar in length and complexity to other lines of PC12 cells (Figure 2A). A potential drawback of the tet system is that there can be a significant basal level of transcription even without doxycycline induction. Indeed, of 80 hygromycin-resistant clones selected after transfection of the parental line with REST cDNA, only one was isolated, called PC12 *tet* REST (clone 1), that exhibited a sufficiently low background level of REST protein before induction (Figure 1A). A different clone (clone 2) is shown in the adjacent lane of the Western blot for comparison of basal levels, but only clone 1 was used in the experiments. REST was present at very low levels in the noninduced state, but levels increased with time after exposure to doxycycline, becoming maximal by 48 hr (Figure 1B). To insure that the induced REST protein was biologically active, PC12 *tet* REST cells were transfected transiently with a Nav1.2 sodium channel CAT reporter gene containing the RE1 sequence. Treatment with doxycycline for 24 hr prior to transfection resulted in a dramatic decrease in CAT activity; and repression was dependent upon the presence of the RE1 (Figure 1C). The lower RE1-mediated CAT activity in PC12 *tet* REST cells compared to the parental PC12 *tet* cells is likely due to a low basal level of REST expression in the absence of doxycycline. Treatment of PC12 *tet* cells with doxycycline did not affect the level of expression of reporter genes containing or lacking the RE1 sequence (Figure 1C).

The NGF induction of Nav1.2 sodium channel mRNA, determined by RNAase protection analysis was blocked completely by expression of REST (Figure 1D). To determine if sustained REST expression would prevent the appearance of membrane excitability, PC12 *tet* REST cells were exposed to doxycycline 24 hr prior to treatment with NGF. Whole-cell recordings performed 48 hr after addition of NGF indicated that the amount of sodium current in the NGF-treated PC12 *tet* REST cells, in the presence of doxycycline, was approximately 3-fold lower than in NGF-treated cells cultured in the absence of doxycycline (Figure 1E).

To test whether REST might also affect other aspects of the neuronal phenotype, we analyzed neurite growth in REST-expressing cells. Figure 2A indicates that neurite extension in NGF-treated PC12 *tet* and PC12 *tet* REST cells was similar in the absence of doxycycline (40.4 ± 0.9 μm compared to 34.3 ± 1.6 μm). However, after induction of REST with doxycycline, neurite length was reduced by approximately 3-fold in the PC12 *tet* REST cells compared to PC12 *tet* cells (16.7 ± 1.6 μm compared to 46.9 ± 8.1 μm). Similar results were obtained using primary mixed murine cultures transduced with an HSV amplicon vector expressing both REST and GFP (Figure 2B). By 72 hr posttransduction, neurons displayed markedly shortened neurites, with preservation of cell somata. The PC12 and the primary neuronal culture data taken together indicate that a program of transcriptional repression of many functionally distinct target genes is likely to be mediated by REST.

Both Repressor Domains of REST Involve HDAC Activity

The N- and C-terminal repressor domains of REST interact with two different corepressors, mSin3A/B and CoREST, respectively. Previous reports have indicated that only the N-terminal repressor domain recruits HDAC activity. These findings raises the question of whether CoREST is required to serve a specific functional role in REST repression, exemplified perhaps by interaction with a unique combination of factors. To address the question of the mechanism of CoREST repression, we first evaluated the efficacy of each repressor domain of REST (Figure 3A). The activity of a reporter gene, containing the Nav1.2 sodium channel promoter and five copies of the Gal4 upstream activator sequence (UAS) in place of the Nav1.2 RE1 (UAS Nav1.2-CAT), was measured as a function of increasing molar amounts of

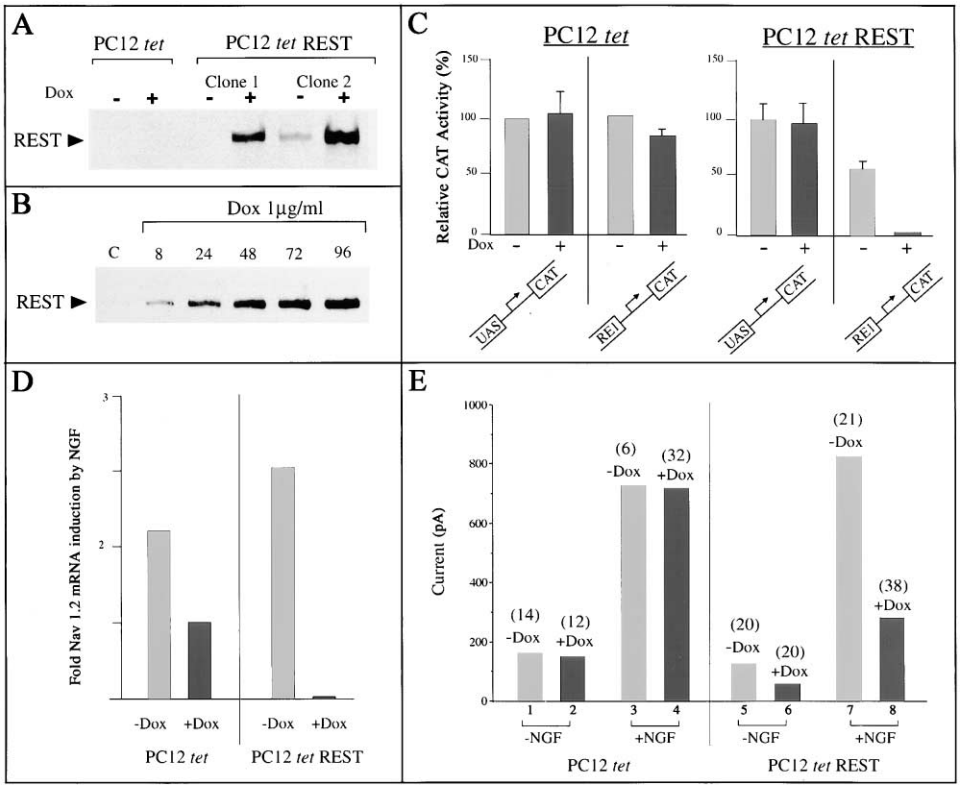

Figure 1. Conditional Expression of REST Results in a Functional Repressor Protein that Interferes with Induction of Sodium Channels by Nerve Growth Factor

(A) Western blot, using an antibody directed against the DNA binding domain of REST, showing induction of REST in two different PC12 clonal lines (PC12 *tet* REST; clone 1 and clone 2) in response to 24 hr treatment with doxycycline (Dox; +). The parental line (PC12 *tet*) does not express REST.

(B) Western blot showing that REST induction by doxycycline is maximal by 48 hr. Cells grown in the absence of doxycycline (C) express minimal levels of REST.

(C) Transient expression analysis indicating that induced REST protein is a functional repressor in PC12 *tet* REST cells, and that repression is dependent upon exposure to doxycycline. Reporter genes contain the sodium channel promoter and either the Nav1.2 sodium channel RE1 (RE1) or five copies of the Gal4 upstream activator sequence (UAS).

(D) RNase protection analysis indicating that REST blocks induction of Nav1.2 sodium channel mRNA by NGF. The indicated PC12 lines were induced with doxycycline 24 hr prior to treatment with NGF for 48 hr.

(E) REST reduces sodium currents. PC12 cells were treated for 24 hr with doxycycline and then exposed to NGF for 48 hr prior to whole-cell recordings. The number of cells analyzed is indicated in parentheses over the histogram bars. Statistical comparisons were performed using a Student's t test. Significant differences of $p < 0.05$ were found for 5 versus 6 and 1 versus 3. Significant differences to a higher level ($p << 0.05$) were found for 7 versus 8 and 5 versus 7. Differences between 1 versus 2 and 3 versus 4 were statistically insignificant ($p > 0.05$).

each repressor domain peptide or of the intact REST protein. The repressor domains were expressed as Gal4-REST fusion proteins, one containing 106 amino acids of the amino-terminal repressor domain (Gal4-N) and the other containing 90 amino acids of the C-terminal repressor domain (Gal4-C). In this assay, both REST repressor domains repressed reporter gene expression equally well (Figure 3A). Moreover, each domain exhibited repressor activity equal to that mediated by the full-length REST protein, at all reporter-to-repressor ratios. The involvement of HDAC with each REST repressor domain was examined. In this experiment, Gal4 proteins containing different portions of REST were evaluated for repressor activity in the presence and absence of the HDAC inhibitor trichostatin A (TSA) (Figure 3B). We observed that repression mediated by REST molecules containing either the C-terminal (Gal4-RESTΔN and Gal4-C) or N-terminal repressor domain (Gal4-RESTΔC and Gal4-N) was relieved dramatically by TSA. Treatment with TSA did not alter reporter activity in the presence of a truncated form of REST lacking both repressor domains (Gal4-RESTΔNΔC). These results suggest that both the amino-and carboxy-terminal repressor domains of REST recruit HDAC.

CoREST Interacts Strongly with HDAC1/2

Because repression by the C-terminal domain of REST was mediated by CoREST, it seemed likely that the TSA sensitivity of the REST domain was due to recruitment of HDAC by CoREST. To test this idea, CoREST complexes were isolated by immunoprecipitation and assayed for the ability to deacetylate chick histones in vitro. Figure 4A shows that there was significant HDAC activity in CoREST complexes from cells transfected with epitope-tagged CoREST (HA-CoREST) as well as in endogenous CoREST complexes (CoREST). HDAC activity was not

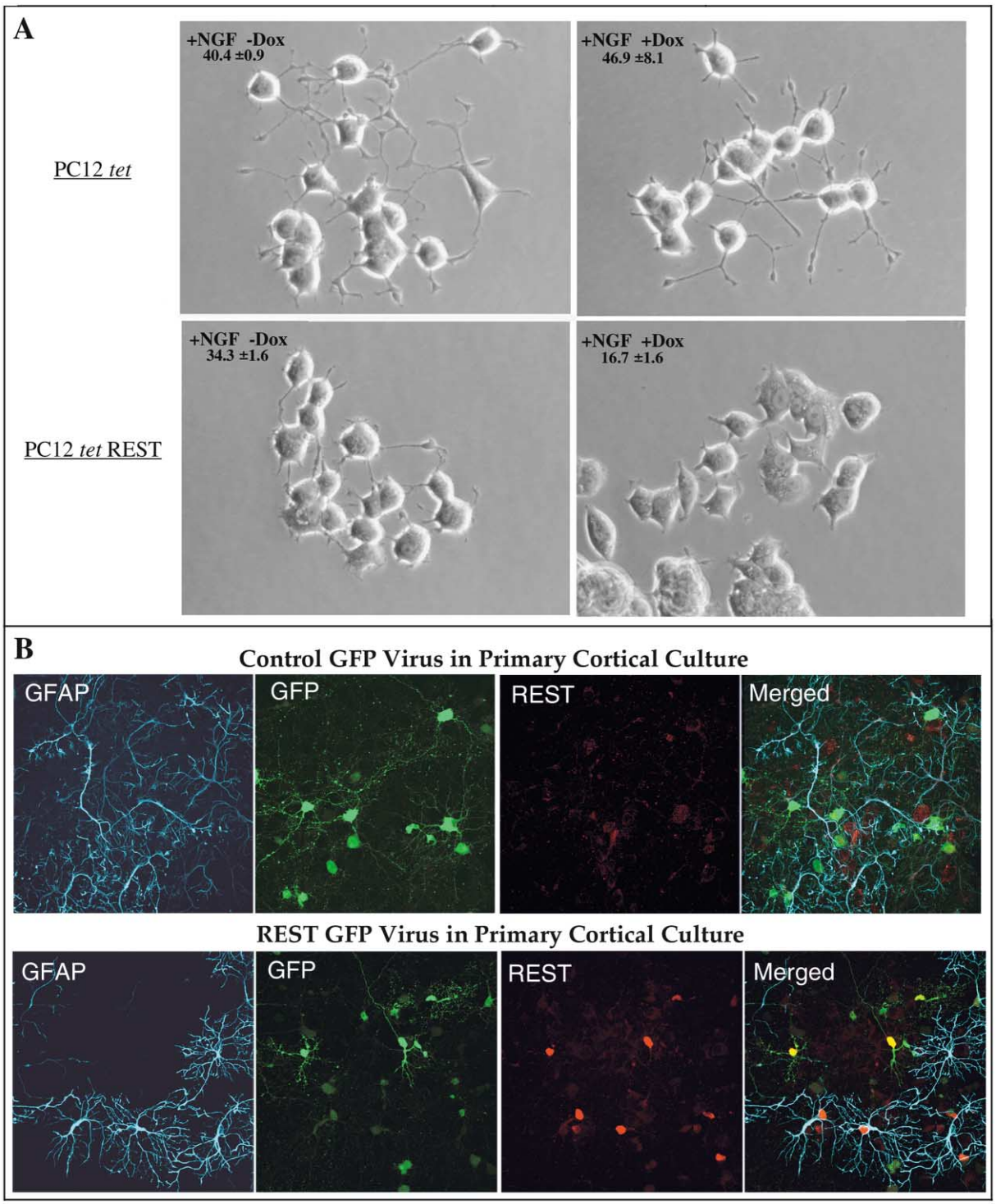

Figure 2. Neurite Growth Is Blocked in Neuronal Cells Expressing REST

(A) REST blocks neurite growth in PC12 cells treated with NGF. The indicated PC12 cell lines were treated as described in Figure 1E. Neurite length, in microns, is shown in the upper left.

(B) Primary neurons expressing REST exhibit shorter and fewer neurites. Mixed cultures of cortical neurons were transduced with a recombinant HSV virus containing GFP or REST-GFP and analyzed 72 hr postinfection. Infected cells express REST in the nuclei (red) and GFP (green). Glial cells are identified by the presence of the GFAP marker (blue).

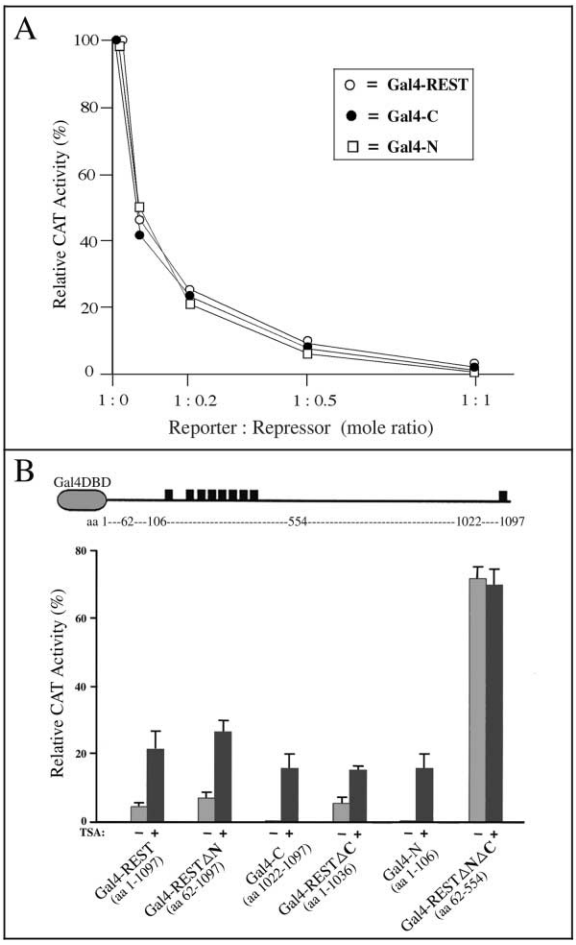

Figure 3. REST Recruits HDAC Activity through Two Distinct Repressor Domains

(A) Each repressor domain in REST represses a reporter gene as well as does the full-length protein. The intact protein (Gal4-REST) or fragments containing the amino-terminal (Gal4-N) or carboxy-terminal (Gal4-C) repressor domains were assayed for ability to repress a UAS Nav1.2-CAT reporter gene at different reporter:repressor molar ratios.

(B) Each repressor domain in REST is associated with HDAC activity. Different portions of REST were fused to the Gal4-DBD and transfected into HEK 293 cells along with a UAS Nav1.2-CAT reporter gene. Transfected cells were incubated for 24 hr in the absence (−) or presence (+) of trichostatin A (TSA).

detected in control experiments using either preimmune IgG or competitor peptide (Figure 4A). To determine which HDAC associates with CoREST, COS 1 cells were immunoprecipitated with anti-CoREST antibodies or were transfected with Myc-tagged CoREST cDNA and immunoprecipitated with antibodies to Myc. The complexes were resolved by SDS-PAGE and Western blotted with antibodies to several HDACs. Reproducible strong signals were obtained only with antibodies to HDAC1 and HDAC2 (Figure 4B). Myc immunoprecipitates from cells transfected with an empty Myc expression vector (α-*myc*) or immunoprecipitates using preimmune sera were always negative (Figure 4B). Neither HDAC3 nor HDAC6 were present in CoREST immunocomplexes obtained from cells cotransfected with Myc-CoREST and either FLAG-HDAC3 or FLAG-HDAC6 (Figure 4B). Interestingly, PC12 cells, which lack REST protein, also contain CoREST/HDAC2 complexes (Figure 4C), suggesting that CoREST recruits HDAC2 independently of REST.

Functional Analysis of the REST Corepressor CoREST

Deletional analysis was performed to identify functional domains in CoREST, including the REST interaction site and any discrete repressor domains in CoREST that could serve as sites of recruitment for HDAC activity. CoREST deletion constructs were confirmed by sequence analysis, and expression levels, determined by Western blots, were similar for all constructs.

The primary structure of the CoREST protein predicts two SANT motifs, motifs that are found in other factors associated with both transcriptional activation and repression (Figure 5). To identify repressor domains, deletions in the N-terminal and C-terminal halves of CoREST were generated and fused to the Gal4 DNA binding domain (Gal4DBD). Repressor activity was determined by cotransfecting Gal4-CoREST deletion constructs with the UAS Nav1.2-CAT reporter, in molar ratios of 1:1 and 5:1 (repressor:reporter). The results summarized in Figure 5A show that, like REST, CoREST contains two distinct repressor domains. Specifically, a domain between amino acids 102 and 195 in the N-terminal half of CoREST (domain 1) and a domain spanning amino acids 321–442, which includes the SANT domain 2, in the C-terminal portion (domain 2) are each sufficient for repression in this assay. However, in contrast to REST, the intact CoREST protein is a much more efficacious repressor than either of its repressor domains (Figures 3 and 5 and data not shown), suggesting cooperativity of these sites in the repression mechanism.

The involvement of HDAC in CoREST repressor activity was examined in HEK 293 cells transfected with Gal4-CoREST fusion cDNAs encoding the repressor domains. The UAS Nav1.2-CAT gene was used as a reporter. Repression was measured in the presence and absence of TSA (Figure 5B). The results indicated that repression by CoREST repressor domains 1 and 2 (Gal4-102–195 and Gal4-291–442, respectively), like that by full-length CoREST (Gal4-CoREST), was relieved by TSA. Treatment with TSA had no effect on reporter activity in the presence of CoREST domains that do not exhibit repressor activity (Gal4-102–180 and Gal4-291–354) (Figure 5B). Because domain 2 does not contain a REST interaction site (Figure 5C), these results suggest that CoREST can recruit HDAC independently of REST and confirm the REST-independent interaction of CoREST/HDAC2 observed in PC12 cells (Figure 4C).

The REST interaction domain in CoREST was mapped by two-hybrid analysis in yeast, using LexA-C-REST as bait in the presence of CoREST deletions fused to the Gal4 activation domain (Gal4AD). Analysis of these deletion mutants using the β-gal reporter revealed two overlapping domains in the CoREST N terminus (aa 102–195 and 145–225) that were sufficient for binding to REST (Figure 5C). The two sites share a core domain of 50 amino acids (145–195) that is necessary but not sufficient to mediate the interaction between REST and

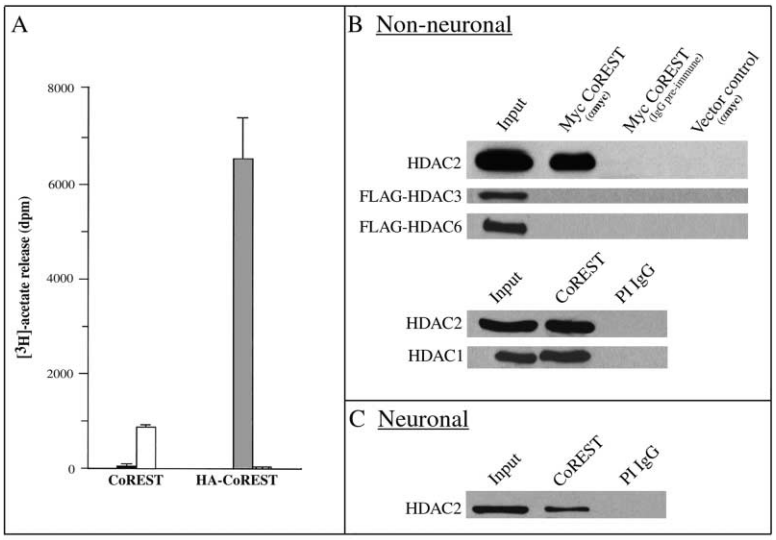

Figure 4. CoREST Is Associated with HDAC In Vivo

(A) HDAC activity is present in CoREST immunocomplexes. Whole-cell extracts from untransformed COS 1 cells were immunoprecipitated with preimmune serum (black bar) or antibodies against CoREST (white bar). Extracts from transfected COS 1 cells were immunoprecipitated with antibodies against the HA epitope (gray bar) or antibodies against the HA epitope in the presence of the blocking peptide (hatched bar).

(B) HDACs 1 and 2 exist in complexes with CoREST. Cells were transfected with Myc-CoREST or cotransfected with Myc-CoREST and FLAG-HDAC3 or Myc-CoREST and FLAG-HDAC6. Extracts were immunoprecipitated with antibodies to Myc or with preimmune IgG. A control transfection was performed using an empty Myc vector (vector control). Parallel experiments were performed for endogenous CoREST, using CoREST antibodies and preimmune IgG as control. The presence of specific HDACs was determined using antibodies to HDAC1, HDAC2, and FLAG.

(C) CoREST forms complexes with HDAC2 in the absence of REST. Whole-cell extracts from PC12 cells were immunoprecipitated with preimmune (PI IgG) or CoREST antibodies and analyzed by Western blot using antibodies to HDAC2.

CoREST. SANT motif I is located within the second interaction domain, suggesting that it contributes to the CoREST/REST interaction.

CoREST and HDAC2 Are Required for Repression by REST

The functional importance of CoREST and HDAC2 in REST-mediated repression was tested by a single-cell microinjection assay in which Rat 1 fibroblasts were injected with a RE1/tk-*lacZ* reporter gene along with antibodies specific for each of the factors (Figure 6A, upper right histogram). The RE1-containing reporter gene was repressed in Rat 1 cells, a result consistent with lack of expression of the endogenous Nav1.2 sodium channel gene and with the presence of an endogenous REST repressor complex (CoREST, mSin3A/B, and HDAC2). Antibodies to REST, CoREST, mSin3A/B, and HDAC2 were each able to relieve this repression. Preimmune IgG had no effect on the level of repression. We next performed experiments to assess the contribution of CoREST to the two individual repressor domains in REST. In these experiments, a UAS/tk-*lacZ* reporter gene was coinjected into Rat 1 cells along with Gal4-REST expression plasmids containing either the N-terminal (Gal4-RESTΔC) or C-terminal (Gal4-RESTΔN) REST repressor domains (Figure 6A). The UAS/tk-*lacZ* reporter gene, in the presence of preimmune antiserum, is transcribed in control Rat 1 cells (Figure 6A). Coinjection with Gal4-RESTΔN or Gal4-RESTΔC resulted in strong repression of the reporter gene (Figure 6A), consistent with the data shown in Figure 3. Microinjection of antibodies to CoREST relieved the repression mediated by Gal4-REST molecules containing the C-terminal repressor domain, but had no effect on repression mediated by the N-terminal repressor domain of REST (Figure 6A). Conversely, microinjection of antibodies to mSin3A/B relieved repression from the N-terminal repressor domain of REST, but not from the C terminus. The amount of derepression was quantified for each microinjection experiment (histograms, Figure 6A). These results indicate that the two repressor domains of REST are distinct and function by recruiting different corepressor complexes.

The role for HDAC2 in each of the CoREST repressor domains was also investigated. For these experiments, Gal4-CoREST containing either domain 1 (Gal4-N-terminus) or domain 2 (Gal4-C-terminus) were injected into Rat 1 cells along with the UAS/tk-*lacZ* reporter gene. Microinjection of anti-HDAC2 antibodies dramatically relieved repression mediated by both CoREST repressor domains (Figure 6B).

The REST Corepressor Complex Occupies the Endogenous Nav1.2 Sodium Channel Gene in Chromatin

To verify that the specific components of the REST complex required for repression in transient assays and identified as complexes in biochemistry assays were also associated with the RE1 sequence in the native sodium channel gene, chromatin immunoprecipitation (ChIP) assays were performed using rat L6 skeletal muscle cells. These cells do not express the Nav1.2 sodium channel gene, but the endogenous gene was derepressed after treatment of the L6 muscle cells with the HDAC inhibitor TSA (data not shown), suggesting that active HDAC was associated with the RE1 in chromatin. Chromatin immunoprecipitation assays with antibodies directed against REST, CoREST, and HDAC2 resulted in an amplified 304 bp DNA fragment corresponding to sequences containing the Nav1.2 sodium channel RE1 (Figure 7A). Antibodies to mSin3A/B also yielded a DNA fragment of the appropriate size in the ChIP assay, indicating the presence of this corepressor on the Nav1.2 sodium channel gene as well. PCR amplification using primers complementary to DNA sequences not related to the RE1 was always negative (data not shown). In

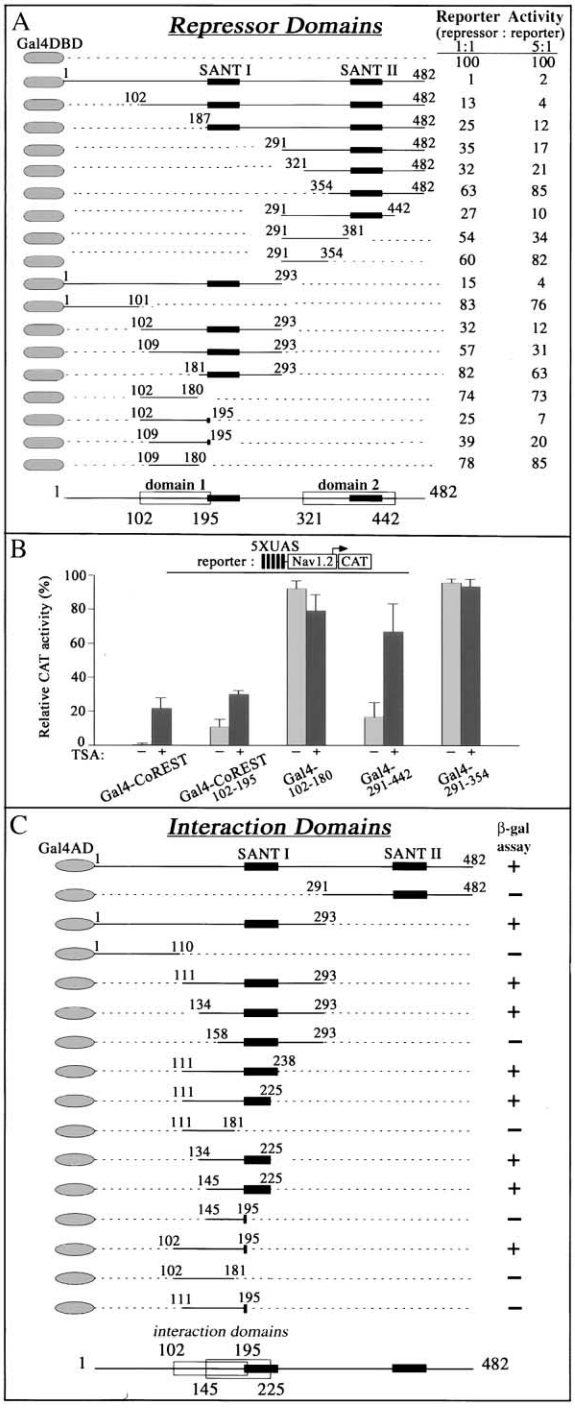

Figure 5. Identification of Functional Domains in CoREST

(A) Deletion analysis resolves two discrete repressor domains in CoREST. CoREST deletion mutants fused to the Gal4DBD are shown with dotted lines indicating the deleted CoREST sequences. HEK 293 cells were cotransfected with the UAS Nav1.2-CAT reporter along with the Gal4DBD-CoREST deletion mutants. Each value represents an average of at least three independent experiments. In the cartoon at the bottom of the panel, the black rectangles indicate the presence of two SANT domains in CoREST.

(B). Repression mediated by the two repressor domains of CoREST is relieved by TSA. HEK 293 cells were cotransfected with the UAS Nav1.2-CAT reporter, and the indicated CoREST deletion mutants fused to the Gal4DBD (A). Cells were either treated with TSA (+, black bars) or nontreated (−, gray bars), followed by determination of CAT activity.

(C) The amino-terminal repressor domain of CoREST maps to the REST interaction domain. Two hybrid interaction results between Gal4AD-CoREST fusion proteins (shown) and the C-terminal half of REST fused to Lex A (LexA-C-REST; residues 525–1097). Dotted lines indicate CoREST sequences deleted from the constructs. The SANT I and II motifs are indicated by black rectangles. The panel on the right shows + or − for β-gal activity for each interaction. The CoREST domains that interact with REST are shown as overlapping rectangles adjacent to SANT domain 1 in the diagram at the bottom of the figure.

contrast to the results obtained in L6 skeletal muscle cells, in PC12 cells, where the Nav1.2 sodium channel gene is expressed, the RE1 sequence was not occupied by REST or by the corepressors CoREST and mSin3A/B (Figure 7A). The presence of the repressor complex on SCG10, another REST-dependent neural-specific gene, was also assessed. Chromatin immunoprecipitation assays indicated that the NRSE/RE1 sequence of the SCG10 gene, like that of the Nav1.2 sodium channel gene, is also occupied by REST, CoREST, and HDAC2 (Figure 7B).

Discussion

Persistent Expression of REST in PC12 Cells Prevents Acquisition of a Terminally Differentiated Phenotype

During normal mouse development, REST is expressed in tissues outside the nervous system and in mitotic neural progenitor cells lining the ventricular zone (Chong et al., 1995; Schoenherr and Anderson, 1995). As the progenitor cells migrate away from the ventricular zone and exit the cell cycle, REST mRNA levels are dramatically decreased and are present at extremely low levels in mature neurons. These observations, along with studies of REST function, have led to the proposal that REST levels must be downregulated in order to permit acquisition of the terminally differentiated neuronal phenotype. If this is true, then overexpression of REST in neuronal precursor cells, which lack REST, should prevent terminal differentiation. The PC12 cell line provided an excellent opportunity to test this prediction. By generating stable lines that express REST under control of an inducible promoter, it was possible to induce REST prior to exposure to a specific growth factor signal (NGF) that cause terminal differentiation. The presence of REST completely blocked induction of Nav1.2 sodium channel mRNA in response to NGF. Additionally, REST caused a statistically significant depression of sodium current in PC12 *tet* REST cells exposed to NGF ($p << 0.05$). A marginal, but statistically significant ($p < 0.05$), decrease in sodium current was also observed in PC12 *tet* REST cells in the presence of doxycycline but in the absence of NGF. This result may indicate that REST also represses the small basal level of sodium current that occurs in the absence of NGF (Mandel et al., 1988).

Consistent with the effects of REST on sodium currents, persistent expression of REST reduced the growth of neurites in response to NGF. Although the specific REST target genes for neurite growth are not known,

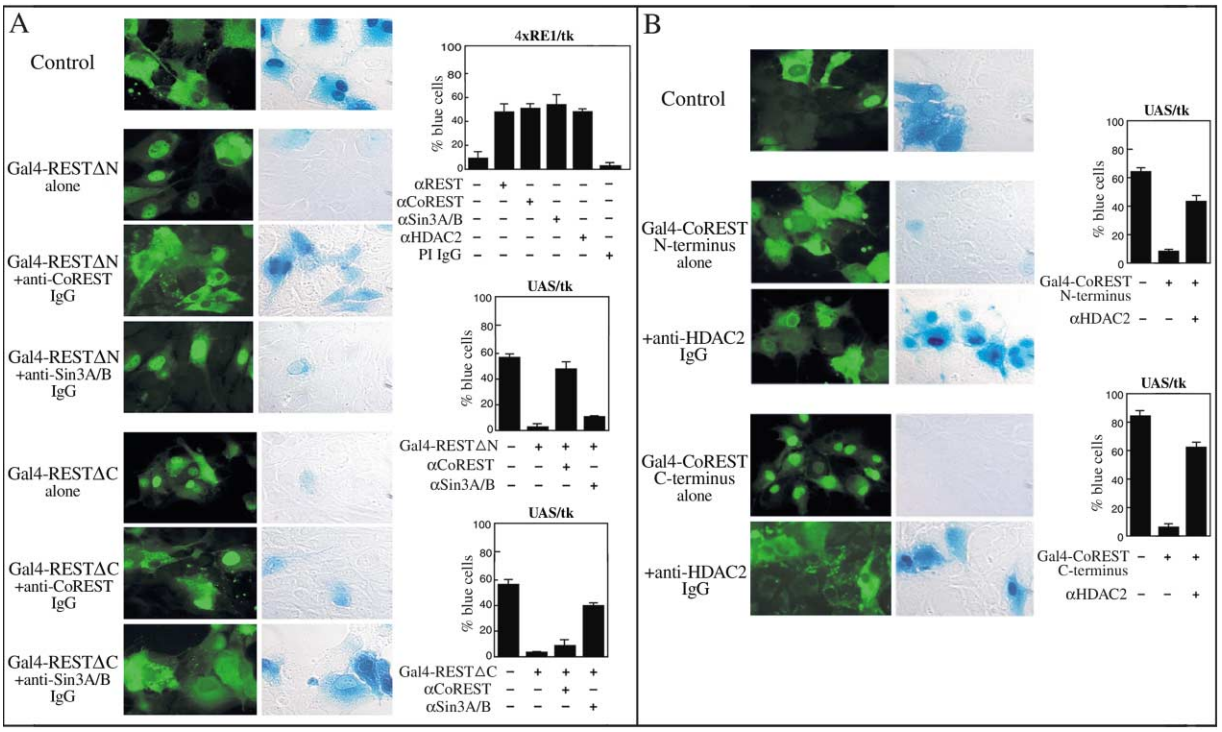

Figure 6. CoREST and HDAC2 Are Required for REST Repression

Reporter genes possessing either four REST binding sites (4xRE1/tk) or three Gal4 binding sites (UAS/tk) fused to *lac*Z were coinjected into the nuclei of nonneuronal cells (Rat 1) with the indicated expression constructs and specific antibodies. Bar graphs indicate the mean value (± SEM) for at least three experiments in which at least 250 cells were injected. Photomicrographs represent typical results. Microinjected cells were identified by fluorescein staining and scored as + or − for the presence of blue Xgal product (phase contrast).

the suppression is consistent with the large number of proteins important to neuronal physiology that are known to be regulated through the REST repressor pathway (Schoenherr et al., 1996). Thus, REST gain-of-function represses neural-specific gene expression and blocks full elaboration of the terminally differentiated phenotype. Taken together with the result that mice harboring deletion of the REST gene show precocious neuronal differentiation (Chen et al., 1998), our study suggests that downregulation of REST is required for both induction and maintenance of a normal neuronal phenotype in vivo.

Repression by REST Requires a New Core Histone Deacetylase Complex

The importance of transcription factors in neuronal differentiation has been underscored by many elegant studies of vertebrate nervous system development (reviewed in Edlund and Jessell, 1999). Indeed, conversion of pluripotent progenitor cells to either neuronal or glial cells can be achieved by forced expression of a single transcription factor (reviewed in Gottlieb and Huettner, 1999). In many cases, however, the molecular mechanism by which these factors lead to gene activation is not known. Moreover, the roles of repressor proteins in guiding neuronal cell fate are much less understood, in general, than are the roles of activator proteins. REST is an excellent model for elucidating molecular mechanisms of repression because many of the target genes are known and RE1/NRSE cognate binding sites have been identified. It is generally accepted that HDACs are important for the repression of many classes of genes, and previous studies have suggested that REST repression also involves HDAC activity. How HDACs are recruited to endogenous REST-dependent genes and to what extent they are important in regulating target genes, however, have remained open questions.

REST contains two independent repressor domains. Previous studies have demonstrated an association of HDAC1 with the amino-terminal repressor domain in REST, mediated by binding to the corepressor mSin3A/B (Grimes et al., 2000; Huang et al., 1999; Naruse et al., 1999; Roopra et al., 2000), and some have suggested, further, that HDAC is associated only with this domain. In the present study, we show that the C-terminal repressor domain in REST also involves HDAC activity. In fact, this result led to an important finding about the type of HDAC required for REST repression. Because mutations in REST that selectively block binding of CoREST to the C terminus also block REST repression (Andres et al., 1999), it seemed likely that REST recruited HDAC via CoREST, rather than by direct binding to HDAC. For this reason, we performed a functional analysis of CoREST. Our analysis revealed that CoREST contains two distinct repressor domains, each of which functions via recruitment of HDAC activity. While each of these two domains is sufficient to repress reporter genes in transient assays, a synergistic effect is observed within the full-length protein, suggesting that the two domains partici-

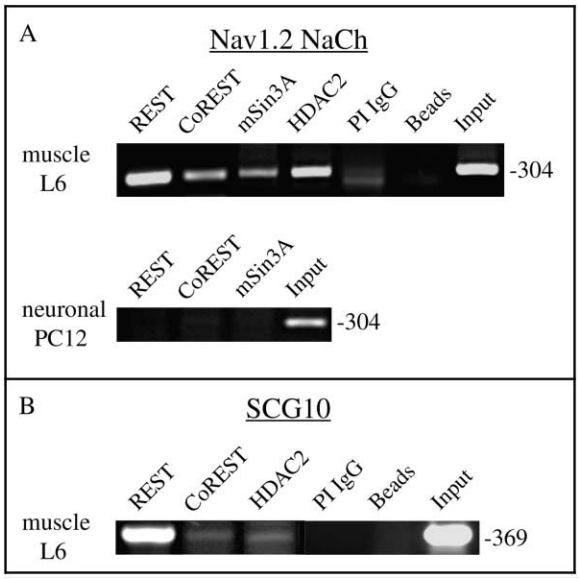

Figure 7. The REST/CoREST Complex Occupies the RE1 Sequence in the Nav1.2 Sodium Channel and SCG10 Genes in Chromatin

(A) Chromatin from rat L6 skeletal muscle cells was crosslinked and immunoprecipitated with antibodies to REST, CoREST, HDAC2, and mSin3A/B. Preimmune antibodies (PI IgG) were used as control in all experiments. The expected size of the "RE1" PCR product (304 bp) is indicated. A parallel experiment was performed using chromatin from PC12 cells that lack REST. Note the absence of PCR product using REST, CoREST, and mSin3A antibodies.

(B) Chromatin immunoprecipitation experiment, using PCR primers specific for the RE1-containing region in the SCG10 gene, and the indicated antibodies. The expected size of the PCR product is shown.

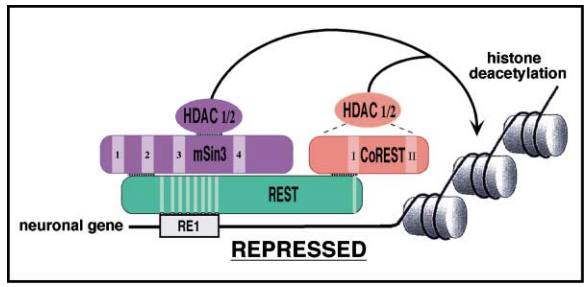

Figure 8. Model of the RE1-Dependent REST Repressor Complex

REST recruits the mSin3 and CoREST corepressors to the RE1 sequence through its repressor domains. CoREST and HDAC1/2 are shown in the same color to indicate that they are tightly associated as a core complex and are likely to interact with DNA binding proteins other than REST. The zinc finger motifs in REST are represented as light-green vertical stripes. The zinc finger in the REST C terminus is required for interaction with CoREST and for repression. The PAH domains in the mSin3 corepressor are indicated by pale vertical lines. Pale vertical stripes in CoREST represent SANT domains 1 and 2.

pate in the same repressor complex. Analysis of the two CoREST repressor domains in PC12 cells indicated that, as in HEK 293 and Rat 1 cells, domain 1 efficiently repressed reporter gene activity, while domain 2 failed to repress in PC12 cells (data not shown). This suggests that domain 1 of CoREST is required for ubiquitous repression, while domain 2 is likely cell type dependent.

Many REST-dependent genes are coordinately repressed in nonneural tissues such as skeletal muscle. The results from our study suggest that the C-terminal repressor domain in REST recruits the same core CoREST/HDAC2 complex to different neural-specific genes. If this were generally true, one prediction would be that in REST knockout animals, several REST-dependent genes might be coordinately derepressed. Unfortunately, deletion of REST in mice by homologous recombination is an embryonic lethal, and the animals die too early for reliable interpretation of gene expression patterns. The availability of mice in which REST or CoREST is conditionally expressed would be very helpful in testing this prediction, as well as in assessing the contribution of CoREST in other repressor pathways.

The apparently stable repression mediated by REST stands in contrast to the ability to switch between repressor and activator complexes characteristic of many DNA binding proteins, such as the steroid hormone receptors and Mad/Max family of factors. Despite this difference, REST and dynamic repressor proteins share the mSin3-HDAC1/2 core corepressor complex. CoREST has been recognized recently as a component of HDAC1/2 immunocomplexes (Humphrey et al., 2001; You et al., 2001); however, it has not thus far been identified as a corepressor for other known repressor proteins. The data, taken together, indicate that the presence of CoREST may be key for long-term tissue-specific repression of neuronal genes mediated by REST. Intriguingly, mSin3A and CoREST are localized differentially during mouse development. At embryonic day 8.5, both REST and mSin3A genes are expressed widely throughout the embryo. CoREST, however, exhibits a much more restricted pattern of expression, being expressed preferentially in the head region (Grimes et al., 2000). By embryonic day 11.5, mSin3A and CoREST are both expressed fairly ubiquitously throughout the embryo (Grimes et al., 2000), suggesting stage-specific recruitment of corepressor complexes.

A Model for REST Repression of Neural-Specific Genes

Based upon our findings, we propose a model for long-term repression of REST-dependent genes (Figure 8). REST binds directly to the specific RE1/NRSE regulatory sequence of the target gene, thereby providing specificity to the repressor complex. There are two repressor domains of REST; one interacts with mSin3A/B (Grimes et al., 2000; Huang et al., 1999; Naruse et al., 1999; Roopra et al., 2000), and the other interacts with CoREST (Andres et al., 1999) (Figure 5). While mSin3 interacts through its PAH1 and PAH2 domains with the N-terminal repressor domain of REST (Grimes et al., 2000), CoREST interacts in part through one of its SANT domains with the REST C-terminal repressor domain (Figure 5). In the case of the Nav1.2 sodium channel gene and other REST-dependent genes, HDAC2 is recruited to REST by CoREST. An interesting question raised by this model is why REST needs two equally efficacious repressor domains. One possibility, mentioned above, is that, in addition to stable repression by REST/CoREST, REST

may also mediate transient expression of genes through interaction with mSin3A/B. In support of this, growth factor modulation of at least two genes has been mapped to an RE1/NRSE sequence (Avisar et al., 1999; Brene et al., 2000).

The apparent molecular weight of CoREST, 66 kDa, is similar to that of HDAC2 (Yang et al., 1996), and both CoREST and HDAC1/2 migrate as doublets on SDS gels. Consequently, the presence of CoREST in purified HDAC2 complexes appears to have been overlooked. In fact, in our coimmunoprecipitation experiments of CoREST and HDAC2, much of the immunoreactivity of HDAC2 is in complexes with CoREST. Moreover, our results in PC12 cells indicate that a stable CoREST/HDAC2 complex exists in the absence of REST. The presence of HDAC1 in CoREST complexes has been confirmed by biochemical purification of the complex (Humphrey et al., 2001; You et al., 2001). Given that CoREST is expressed at high levels in neurons and that CoREST itself functions as a repressor in the absence of REST (Andres et al., 1999), our findings suggest that the CoREST/HDAC2 complex plays an important role in repressor pathways in differentiated neurons. The precise role for this complex awaits identification of the neuronal DNA binding partners for CoREST.

Experimental Procedures

Plasmids

The FLAG-HDAC expression constructs were kindly provided by Drs. Stuart Schreiber, Edward Seto, and Wen-Min Yang. All Nav1.2 sodium channel reporter constructs, as well as the REST cDNA expression plasmids Gal4-REST (Gal4-REEX1), Gal4-RESTΔN (Gal4-REEX8), Gal4-RESTΔNΔC (Gal4-p73), and Gal4-C (Gal4-C3) have been described previously (Maue et al., 1990; Tapia-Ramirez et al., 1997). Gal4-RESTΔC was constructed by replacing the C-terminal ClaI/XbaI fragment of Gal4-REST (residues 226–1097) with the ClaI/XbaI C-terminal fragment of REEX9 (residues 226–1036) (Tapia-Ramirez et al., 1997). Gal4-N was generated by subcloning a PCR fragment containing the N-terminal sequences of REST (residues 1–106) in frame with Gal4-DBD in the pSG424 vector. LexA-C-REST, containing amino acid residues 525–1097 of REST, was described before (Andres et al., 1999). CoREST deletions generated by PCR were fused in frame to the Gal4-DBD in pSG424 or to the Gal4-AD in pGAD GH (Andres et al., 1999). HA-tagged CoREST was constructed by fusing a CoREST fragment coding for amino acids 102–482 to the HA epitope tag in the expression vector pCGN (Andres et al., 1999). Myc-tagged CoREST was described previously (Andres et al., 1999).

The inducible REST expression vector pTRE-REST, containing the FLAG epitope, was generated as follows: a FLAG-tagged REST cDNA in pCMV-Tag1 (Stratagene) was described previously (Grimes et al., 2000). The intact FLAG-REST cDNA was subcloned into pTRE (Clontech) at the EcoRI site using standard cloning techniques. The pTK-Hyg plasmid was purchased from Clontech.

The pHSVPrPUC/CMVegfp amplicon, which expresses the enhanced green fluorescent protein (eGFP) gene under the transcriptional control of the cytomegalovirus (CMV) immediate early (IE) promoter, has been described previously (Bowers et al., 2001). The pHSVrest/CMVegfp amplicon was constructed by inserting a cDNA encoding REST into the parent amplicon pHSVPrPUC/CMVegfp. The final vector encodes REST under transcriptional control of the HSV IE4/5 gene promoter and eGFP under control of the CMV IE promoter.

Cell Culture and Transient Transfection Analyses

HEK 293 cells, Rat 1 fibroblasts, L6 cells, and COS 1 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine calf serum. PC12 cell lines (PC12, PC 12 *tet,* and PC12 *tet* REST) were maintained in DMEM supplemented with 10% horse serum and 5% fetal calf serum. For PC12 *tet* and PC12 *tet* REST, the antibiotic Geneticin or Geneticin plus Hygromicin B, was added to the media of the indicated cell lines, respectively, at 100 μg/ml. Antibiotics were not present during experiments. Transfection of HEK 293, COS 1, and PC12 cell lines was performed in 10%–20% confluent cultures in 100 mm dishes, using the FuGENE 6 transfecting reagent (Roche, Inc.). In all transfections, the amounts of plasmid DNA were adjusted on a molar basis, and the total quantity of DNA in the transfection mixture was kept constant by the addition of pBluescript (Stratagene, Inc.) to a total of between 10 and 12 μg DNA.

For electrophysiological and morphological studies, 10% confluent PC12 *tet* or PC12 *tet* REST cells, in 35 or 60 mm dishes, were treated with 1 μg/ml doxycycline or left untreated. After 24 hr, NGF (100 ng/ml) was added for an additional 48 hr.

For repression assays in HEK 293 cells, 2 μg of the UAS Nav1.2-CAT reporter was transfected along with Gal4-CoREST cDNA, or its deletion mutants, in reporter to repressor molar ratios of 1:1 or 1:5. For experiments using trichostatin A (TSA), HEK 293 cells were transfected with 5 μg of the reporter gene alone, or along with Gal4REST or Gal4-CoREST deletion constructs, in molar ratios of 1:1 (reporter:Gal4-REST construct) or 1:3 (reporter:Gal4-CoREST construct). Sixteen hours after transfection, cells were treated with 100 ng/ml TSA for an additional 24 hr.

For repression assays in the PC12 *tet* and PC12 *tet* REST cell lines, 1 μg/ml doxycycline was added 24 hr prior to transfection. The doxycycline was left in the culture medium until harvesting for the assay measurements. The constructs UAS Nav1.2-CAT or RE1 Nav1.2 CAT (7 μg each) were transfected as described previously.

All CAT assays were performed on 15 μg protein extracts, obtained from cells harvested 40 hr after transfection, as described (Maue et al., 1990).

Generation of the PC12 *tet* REST Stable Cell Line

PC12 *tet* cells, stably transfected with the tetracycline transactivator rtTA (Tet-On), were purchased from Clontech. Cells were cotransfected with 20 μg of pTRE-REST and 2 μg of pTK-Hyg using electroporation and then plated for 48 hr. Hygromycin B (200 μg/ml) was added for selection, and positive clones were picked and plated with or without 1 μg/ml of doxycycline for 48 hr. Nuclear protein extracts were obtained as described (Grimes et al., 2000) and analyzed by Western blotting for inducible expression of REST protein. Anti-FLAG (Sigma) or anti-REST (Chong et al., 1995) specific antibodies were used for detection.

Primary Murine Cortical Cultures and Viral Transduction

Primary mixed cortical neuronal cultures were established from E15.5 mouse (C57/BL6) embryos (Halterman et al., 1999) and helper virus-free amplicon packaging, and virus purification was performed as previously described (Bowers et al., 2001). Amplicon virus numbers were determined by assessing both expression and transduction titers as previously described (Bowers et al., 2000).

Cultures were treated with 10 μM cytosine arabinoside (Sigma) after 4 days in vitro (4 DIV) to impede glia proliferation. Infection of cultures was performed on 6 DIV by reducing volume to 300 μl, adding 5–10 μl virus (MOI of 0.5) for 1 hr at 37°C then adding 300 μl of conditioned media (removed prior to infection). Cells were fed every 3 days by 50% media replacement, if necessary.

Immunocytochemistry of Primary Cortical Cultures

Primary cortical cultures transduced with GFP or GFP-REST expressing viral vectors were fixed in 4% paraformaldehyde in PBS at 4°C, permeableized in 0.1% Triton-X 100, and blocked in 2% nonfat dry milk and 10% normal goat serum. The primary antibodies used were a mouse monoclonal anti-glial fibrillary acidic protein (GFAP, Dako) and a rabbit polyclonal p73 REST antibody (Chong et al., 1995). After rinsing in PBS, cells were incubated in goat anti-mouse CY5 (Jackson Immuno Research) and goat anti-rabbit Alexa 568 (Molecular Probes). Images were collected in "z" series on a Zeiss confocal LSM 510 microscope.

Electrophysiology

Measurements of sodium current from the PC12 *tet* and PC12 *tet* REST cell lines were made using the ruptured whole-cell recording

technique. Recording pipettes were pulled to a tip outer diameter of 3 μm and lightly fire polished immediately prior to use. The pipettes were filled with a solution containing (in mM) 140 CsCl, 1 Cs-EGTA, and 10 Cs-HEPES, with pH adjusted to 7.2 with CsOH. The bath solution contained (in mM) 140 NaCl, 1KCl, 0.1 CaCl2, and 10 Na-HEPES. Cells were held at −100 mV in order to remove all steady state inactivation of sodium channels. Sodium currents were elicited with 10 ms step depolarizations. The resultant current was recorded using an Axopatch 200B amplifier, filtered at 5 kHz, and digitized at 10 kHz prior to analysis. The potential corresponding to peak current was determined from the current-voltage relations, and the leak-corrected (P/10) sodium current amplitude at this potential was determined.

Ribonuclease Protection Assay (RPA)

PC12 *tet* or PC12 *tet* REST cells (10% confluency) were treated with 1 μg/ml doxycycline for 24 hr. NGF (100 ng/ml) was added for another 48 hr. RNA was isolated as described (Cathala et al., 1983). RPA was performed on 25 μg total RNA using the Ambion RPA III kit. Hybridization was performed using a Nav1.2 sodium channel-specific probe (Cooperman et al., 1987), and cyclophilin (Ambion) was used as an internal probe to standardize mRNA levels.

Immunoprecipitation and HDAC Assay

Immunoprecipitations for HDAC assays were performed as described (Laherty et al., 1997). Briefly, COS 1 cells were transfected with 7 μg of HA-CoREST construct and harvested 48 hr after transfection. Normal and transfected COS 1 cells were lysed in a low-stringency buffer (PBS with 0.1% NP-40 and protease inhibitors), sonicated, and centrifuged. Cell extracts were precleared using protein A-sepharose CL4B beads (Sigma) and immunoprecipitated with preimmune antiserum, anti-HA (Santa Cruz), or anti-CoREST antibodies (Andres et al., 1999), with or without the HA-blocking peptide as indicated in individual experiments. Protein A-sepharose bead-bound complexes were washed four times in low stringency buffer, flash-frozen in HDAC buffer (20 mM Tris [pH 8.0], 150 mM NaCl, 10% glycerol) and stored at -80°C until use.

HDAC assays were performed as described (Hendzel et al., 1991). Briefly, immunoprecipitated complexes were incubated for 60 min at 37°C in 25 mM sodium phosphate/citric acid (pH 7.0) buffer containing 500 μg acid-soluble [^{3}H]-acetate-labeled chicken erythrocyte histone. The reaction was terminated upon addition of 0.12 N acetic acid and 0.72 N HCl. The released [^{3}H]-acetate was extracted with ethyl-acetate and quantified by scintillation counting. The assays were performed in triplicate, and the nonenzymatic release of label was subtracted.

Coimmunoprecipitation and Western Blot Analysis

Whole-cell lysates were obtained from PC12 cells or COS 1 cells (transfected or untransfected) as follows: cells were washed twice with PBS and lysed for 30 min at 4°C in low-stringency buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.5% Triton X-100, 10% glycerol, 0.5 mM DTT, and complete protease inhibitors (Roche). Lystes were centrifuged at 4°C for 15 min at 14 K, and the supernatants (whole-cell extracts) were precleared by incubation with protein G-Sepharose CL4B (Sigma) for 1 hr. Immunoprecipitates were obtained by incubating the cleared extracts with the specific antibodies for 16 hr followed by 2 hr incubation with protein G-Sepharose. Bead-bound complexes were washed four times with washing buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.1% TritonX-100, and 5% glycerol) and boiled in SDS-loading buffer. Western blot analysis was carried out using standard techniques. Antibodies to Myc and HDAC2 were purchased from Zymed. Antibodies to the FLAG M2 epitope were from Sigma, and antibodies for HDAC1 from ABR. CoREST antibodies were previously described (Andres et al., 1999).

Yeast Two-Hybrid Assay

The LexA-C-REST fusion construct was used as bait. Deletion constructs of CoREST fused to the Gal4AD were analyzed for their interaction with the bait using *HIS3* or *lacZ* reporter gene expression assays in the yeast strain L40 as described (Park and Sternglanz, 1998).

Single-Cell Microinjection Assay

Single-cell gene expression experiments were conducted essentially as described (Heinzel et al., 1997). DNA was microinjected into the nuclei of Rat 1 cells at a concentration of 100 μg/ml in the presence/absence of coinjected IgG directed against mSin3A/B, CoREST, or HDAC2. Injected cells were unambiguously identified by coinjection of fluorescein-conjugated dextran, Mr 70,000 (Molecular Probes). Quantitation was accomplished after Xgal staining by scoring each injected cell as positive or negative for any degree of blue staining. Approximately 250 cells were injected in each experiment, and each assay was done at least three times.

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed according to the Upstate Biotechnology protocol. Briefly, L6 cells from a 100 mm dish (~70% confluent) were treated with 1% formaldehyde for 10 min at room temperature. Cells were lysed in buffer containing 5 mM HEPES (pH 8.0), 85 mM KCl, and 0.5% Triton. Isolated nuclei were resuspended in 500 μl of nuclei lysis buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS and sonicated under conditions that reduced DNA length to between 200 and 1000 bp. Nuclear extracts were precleared for 1 hr at 4°C using protein G agarose beads (GIBCO, Inc.) preadsorbed with sonicated single-strand DNA. The chromatin suspension (100 μl) was diluted 10-fold in IP buffer (Upstate Biotech, Inc.) and incubated with 5 μg of specific antibodies for 12 hr at 4°C. Immunocomplexes were collected on protein G-agarose beads preadsorbed with sonicated single-strand DNA. Following washes and elution, cross-linked complexes were reversed by heating at 65°C for 12 hr. DNA was treated with proteinase K, extracted with phenol-chloroform, precipitated, and resuspended in 30 μl of TE. The DNA sequences in the immunoprecipitates were detected by PCR, using the following sets of primers designed to amplify the RE1-containing regions of the specific neuronal genes: Nav1.2, (5′ primer) AAGCTTGGACACTCCAGGAGAGCCTG; (3′ primer) CGAAG TGAAAGCCTGTTTAGGAGGGTAGG; SCG10, (5′ primer) AAACCCT GCCATTTCCATACAGTACG; (3′ primer) AACTCATGTAGGAATTGT GGTTGAGG.

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