THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 13, Issue of March 31, pp. 9461–9467, 2000

Printed in U.S.A.

The Co-repressor mSin3A Is a Functional Component of the REST-CoREST Repressor Complex*

(Received for publication, September 8, 1999, and in revised form, November 16, 1999)

Julia A. Grimes‡§¶, Søren J. Nielsen§‖, Elena Battaglioli‡¶, Eric A. Miska‖, Joan C. Speh‡, Dianna L. Berry‡ ‡‡, Fouad Atouf‡¶, Bernadette C. Holdener‡‡, Gail Mandel‡§§, and Tony Kouzarides‖**

From the ‡Howard Hughes Medical Institute and Department of Neurobiology and Behavior and the ‡‡Department of Cell Biology and Biochemistry, State University of New York, Stony Brook, New York, 11794-5230 and the ‖Wellcome/Cancer Research Campaign Institute and Department of Pathology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, United Kingdom

The repressor REST/NRSF restricts expression of a large set of genes to neurons by suppressing their expression in non-neural tissues. We find that REST repression involves two distinct repressor proteins. One of these, CoREST, interacts with the COOH-terminal repressor domain of REST (Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallmanm J., Ballas, N., and Mandel, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 9873–9878). Here we show that the co-repressor mSin3A also interacts with REST. The REST-mSin3A association involves the NH_2-terminal repressor domain of REST and the paired amphipathic helix 2 domain of mSin3A. REST forms complexes with endogenous mSin3A in mammalian cells, and both mSin3A and CoREST interact with REST in intact mammalian cells. REST repression is blocked in yeast lacking Sin3 and rescued in its presence. In mammalian cells, repression by REST is reduced when binding to mSin3A is inhibited. In mouse embryos, the distribution of mSin3A and REST transcripts is largely coincident. The pattern of CoREST gene expression is more restricted, suggesting that mSin3A is required constitutively for REST repression, whereas CoREST is recruited for more specialized repressor functions.

Many genes essential for neuronal functioning, including the brain type II voltage-dependent sodium channel, neuronal growth factors, and neurotransmitter receptors, are repressed in non-neuronal cells by the transcriptional repressor, REST/NRSF (2–5). Removal of the REST/NRSF binding site (RE1/NRSE) from the regulatory region of transgenes (6) or expression of a dominant negative form of REST/NRSF *in vivo* (5) results in aberrant expression of the target genes in non-neural tissues. Deletion of the mouse REST/NRSF gene by homologous recombination results in embryonic lethality (5). Because of its importance in establishing and maintaining the expression pattern of a large number of genes required for neuronal functioning, it was of interest to identify the molecules involved in the repressor mechanism.

Previous studies identified two distinct domains in the NH_2 and COOH termini of REST that were both necessary and sufficient to repress brain type II sodium channel reporter genes in transient transfection assays (7) and showed that repression by each of these two domains required distinct titratable factors (8). Recently, repression from the COOH-terminal domain was determined to be mediated by the co-repressor, CoREST (1). We sought to determine whether, in addition to CoREST, mSin3A, a co-repressor for several regulated repressor complexes, might also be involved in REST repression. We found that mSin3A is indeed a functional co-repressor for REST. mSin3A interacts with REST *in vitro*, in yeast and in intact mammalian cells, and interestingly, the binding site maps to the NH_2-terminal repressor domain in REST. Furthermore, experiments both in yeast and mammalian cells showed that mSin3A is involved in repressor function. *In vivo*, the expression patterns of the co-repressors mSin3A and CoREST are distinct. Specifically, in early embryogenesis CoREST exhibits a much more restricted pattern of expression compared with REST and mSin3A, suggesting that the composition of the REST repressor complex during development is dynamic.

EXPERIMENTAL PROCEDURES

Plasmid Constructions

Yeast Two-hybrid Constructs—LexA-mSin3A was obtained by cloning full-length mSin3A into pBTM116 by standard PCR[1] cloning techniques. pGADmSin3A was made by cloning full-length mSin3A (provided by Carol Laherty and Bob Eisenman, Fred Hutchinson Cancer Research Center, Seattle, WA) into pGADGH (donated by Rolf Sternglanz, SUNY, Stony Brook, NY) using standard PCR-based cloning techniques. pGADNmSin3A was obtained by cloning a restriction fragment encoding the first 530 amino acids of mSin3A into pGADGH. To make a fusion between full-length CoREST and the LexA DNA binding domain, a 3030-base pair *Nae*I–*Pst*I fragment from pcDNA1.1CoREST (1) was cloned between the *Sma*I and *Pst*I sites of the vector pBTM to generate pBTMCoREST. A fusion of the LexA DNA binding domain to amino acids 102–482 of CoREST has been described previously (1). A fusion of the LexA DNA binding domain to amino acids 1–293 of CoREST was made by cloning an *Eco*RI/*Eco*RI fragment of Gal4-CoREST (amino acids 1–293)[2] into pBTM. Fusions between the LexA DNA binding domain and the amino or carboxyl portions of REST (N-REST and C-REST, respectively) have been described previously (1).

* This work was supported by a grant from the McKnight Foundation and National Institutes of Health Grant NS22518 (to G. M.) and Cancer Research Campaign (CRC) Grant DR11 (to T. K.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These two authors contributed equally to this work.
¶ Postdoctoral Associate in the Howard Hughes Medical Institute.
** Supported by a CRC studentship.
§§ An Investigator in the Howard Hughes Medical Institute. To whom correspondence should be addressed. Tel.: 631-632-8929; Fax: 631-632-9714; E-mail: gmandel@notes.cc.sunysb.edu.

[1] The abbreviations used are: PCR, polymerase chain reaction; PAH, paired amphipathic helix; RE1, repressor element 1; GST, glutathione *S*-transferase; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PBST, PBS-Triton X-100; WT, wild type; N-REST and C-REST, NH_2-terminal and COOH-terminal portions of REST, respectively.

[2] N. Ballas, unpublished.

GST Fusion Constructs for Bacterial Expression—GST-REST-(32–122) was made by subcloning an *Eco*RI/*Bam*HI fragment of VP16 REST (obtained in the two-hybrid screen) into pGEX-3X.

Mammalian Expression Constructs—An NH_2-terminal FLAG epitope was added to REST by cloning it into pCMV-Tag1 (Stratagene) by standard PCR-based cloning techniques. To generate RESTΔNLS FLAG, nucleotides 1535–1566 (encoding the consensus NLS) of REST-FLAG were removed and replaced with a *Bam*HI site by PCR, and the RESTΔNLS FLAG coding sequence was subcloned into pcDNA3.1+ (Invitrogen). The pTet-On plasmid was purchased from CLONTECH, and the tetracycline-inducible REST expression vector, pTRE-REST, was made by cloning REST-FLAG into pTRE (CLONTECH) at the *Eco*RI site. To generate REST Myc, the coding sequence of REST was cloned into pcDNA3.1(−) Myc-His A (Invitrogen) by standard PCR-based cloning techniques. mSin3A Myc was generously donated by Carol Laherty and Bob Eisenman. CoREST Myc has been described previously (1). The RE1-containing type II sodium channel reporter gene, pSDK7, has been described previously (9).

In Situ Hybridization Probes—A plasmid containing a 1.1-kilobase pair cDNA fragment spanning the zinc finger DNA binding domain of mouse REST (mREST in pBluescript) has been described previously (2). Linearization of this plasmid with *Eco*RI and transcription with T7 RNA polymerase were used to generate antisense transcripts, while digestion with *Kpn*I and transcription with T3 RNA polymerase were used to generate sense transcripts. A mouse expressed sequence tag containing a 1.3-kilobase pair insert spanning the entire open reading frame of CoREST (identified by homology with human CoREST) was purchased from Genome Systems Inc. Linearization with *Bam*HI and transcription with T7 RNA polymerase yielded the antisense transcript, while digestion with *Xho*I and transcription with T3 RNA polymerase yielded a sense transcript. An 800-base pair *Nsi*I/*Hin*dIII fragment spanning amino acids 274–533 of mSin3A was cloned into pBluescript II KS. Antisense and sense riboprobes were transcribed from plasmids linearized with *Spe*I or *Eco*RI, using T7 and T3 RNA polymerases, respectively.

All fusion protein constructs were sequenced across the junction to ensure that inserts were in frame. All constructs generated by PCR were sequenced completely.

Yeast Two-hybrid Assay

pBTM116-Sin3A was transformed into L40 yeast together with a mouse 9.5–10.5 days postcoitum cDNA library ligated into pVP16 as described (10). An estimated 5×10^6 transformants were screened. Proteins interacting with mSin3A were identified by growth on selective medium in the presence of 5 mM 3-aminotriazole and confirmed by assaying for β-galactosidase activity. Plasmids rescued from positive yeast colonies were retransformed into L40 together with either pBTM116-mSin3A or pBTM116-lamin to assess the specificity of the interaction. Positively interacting clones were characterized by sequence analysis. Direct yeast interaction assays were performed using yeast transformed with either LexA-REST, LexA-NREST, or LexA-CREST and either pGAD-mSin3A, pGAD-NmSin3A, or pGAD-CoREST. Activity of *HIS3* and *LacZ* reporter genes was used to identify positive interactions.

GST Pull-down Assays

Expression and purification of GST-fusion proteins and GST pull-down assays were performed as described (11). *In vitro* transcription/translation was performed using the rabbit reticulocyte lysate-based TnT system from Promega according to the manufacturer's instructions. The *in vitro* translatable mSin3A deletion series in pCS2 + MT was kindly provided by Carol Laherty and Bob Eisenman.

Co-immunoprecipitations and Western Blotting

HEK-293 cells were grown as described previously (1) and transfected with pTetOn and pTRE-REST-FLAG using Fugene transfection reagent (Roche Molecular Biochemicals). The tetracycline analogue doxycycline was added to the medium to a final concentration of 2 μg/ml to induce expression of REST. Cells were harvested 48 h after transfection. Cell pellets were resuspended in buffer H (20 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml antitrypsin, aprotinin, and leupeptin) and passed through a 25-gauge needle five times. Following centrifugation, the nuclear pellet was resuspended and rotated for 20 min at 4 °C in buffer C (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml antitrypsin, aprotinin, and leupeptin). After centrifugation, the supernatant consisting of nuclear proteins was diluted to create immunoprecipitation buffer (20 mM HEPES-KOH, pH 7.9, 8.9% glycerol, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 2 μg/ml antitrypsin, aprotinin, and leupeptin). 400-μg aliquots of nuclear extract were precleared by rotating at 4 °C for 5 h with 20 μl of protein G-agarose beads (Life Technologies, Inc.) and 5 μg of preimmune rabbit IgG. Nuclear extracts were then recovered by centrifugation and added to 20 μl of fresh protein G-agarose beads and 5 μg of the appropriate polyclonal antibodies for immunoprecipitation. The antibodies used were anti-REST (2), anti-CoREST (1), anti mSin3A K-20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-mSin3A AK-11 (Santa Cruz Biotechnology), and preimmune rabbit IgG prepared using the Avidchrom protein A kit (Sigma). Samples were immunoprecipitated by rotation overnight at 4 °C. Beads were washed 3 times in high salt immunoprecipitation buffer (250 mM NaCl), and bound proteins were eluted by boiling in Laemmli sample buffer with 10% β-mercaptoethanol. Immunoprecipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis, and Western blotting was performed using a monoclonal anti-FLAG M5 antibody (Sigma) and the K-20 mSin3A antibody.

Indirect Immunofluorescence and Confocal Microscopy

COS-1 cells were cultured as described previously (7). After plating on poly-L-lysine-coated glass coverslips, cells were transfected with FLAG and Myc epitope-tagged expression constructs using Fugene transfection reagent (Roche Molecular Biochemicals). 48 h after transfection, cells were washed twice in phosphate-buffered saline (PBS) and fixed in 3% paraformaldehyde-PBS for 10 min. Cells were then washed four times in PBS, permeabilized for 10 min in 0.1% Triton X-100-PBS (PBST), and incubated for 1 h in 5% nonfat powdered milk in PBST. Coverslips were then incubated for 1 h in anti-FLAG M5 monoclonal antibody (Sigma) and anti-Myc polyclonal antibody (Upstate Biotechnology Inc.), both at a 1:500 dilution. Cells were washed four times in PBST before incubating in Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, Inc., Eugene, OR) and Alexa 546-conjugated goat anti-rabbit antibody (Molecular Probes), both at a dilution of 1:1000, for 1 h in the dark. Coverslips were then washed four times in PBST and once in PBS and mounted in Vectashield H-1000 (Vector Laboratories, Inc.). Cells were viewed using a Zeiss LSM 510 laser-scanning confocal microscope.

Yeast Reporter Gene Assays

Yeast wild type (WT) and Sin3 deletion strains (DY1641 and DY2516, respectively (12)) were transformed with pMN-REST, pRS313-Sin3 (M1635; Ref. 13), or the corresponding empty expression constructs. Both yeast strains contain an integrated *LexA-LacZ* reporter so that repression by Lex-REST expressed from pMN-REST can be assessed by monitoring β-galactosidase activity.

Mammalian Reporter Gene Assays

HEK293 cells were grown as described previously (1) and transfected using Fugene (Roche) according to the manufacturer's instructions. Cells were transfected with 2 μg of the type II sodium channel CAT reporter gene, pSDK7. Cells were also transfected with either 0.5 μg of the "competitor" Gal4F4 or an equimolar amount of the empty Gal4 vector and either 0.5 μg of mSin3A or an equimolar amount of the empty vector pCS2 + MT. Cells were harvested 48 h after transfection. CAT assays were performed as described previously (7).

In Situ Hybridization

Digoxygenin-labeled sense and antisense riboprobes were generated using a digoxygenin RNA labeling kit (SP6/T7; Roche Molecular Biochemicals), treated with DNase, and purified on G50 Sepharose columns. Embryos were collected from CD-1 mice at embryonic days 8.5 and 11.5. Whole mount embryos were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in PBS with 2 mM EGTA, "bleached" in 6% H_2O_2 in PBS with 0.1% Tween 20, and stored at −20 °C in absolute methanol until used for hybridization. Embryos for paraffin embedding were fixed in Bouin's solution, dehydrated, cleared, embedded in Paraplast, and serially sectioned at 8 μm.

Whole mount hybridization was carried out as described previously (14). Paraffin-sectioned material was hybridized with a modification of the method of Hockfield *et al.* (15). In brief, sections were deparaffinized, hydrated, proteinase K (10 μg/ml in PBS with Tween)-treated, fixed in 4% paraformaldehyde, acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine, dehydrated, and dried before hybridization.

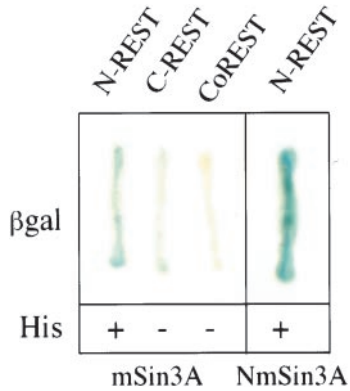

FIG. 1. **The NH_2 terminus of REST interacts with the NH_2 terminus of mSin3A in yeast.** Shown is a yeast two-hybrid assay to test the interaction between full-length mSin3A or the NH_2-terminal half of mSin3A (NmSin3A) with either the NH_2 terminus of REST (N-REST), the COOH terminus of REST (C-REST), or CoREST. Activity of the β-galactosidase reporter gene is visualized as a *blue* reaction product in streaks of yeast cells (*upper panel*). Activity of the His reporter gene enabling growth on medium lacking histidine is indicated by the *plus symbol* (*lower panel*). Activity of both reporter genes indicates a positive interaction between two proteins.

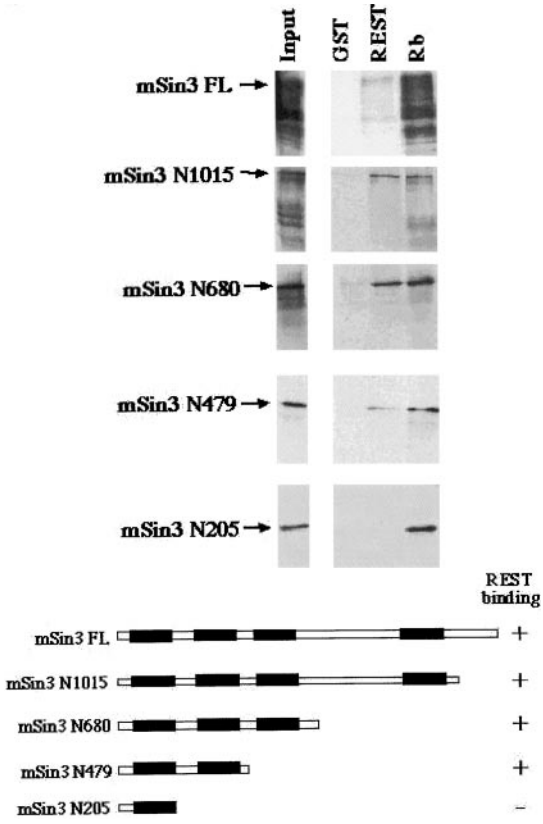

FIG. 2. **The NH_2 terminus of REST interacts directly with the PAH-2 domain of mSin3A.** *A*, GST pull-down assay in which ^{35}S-labeled *in vitro* translated full-length mSin3A (mSin3A FL) or COOH-terminal deletions to amino acid residues 1015 (*mSin3A N1015*), 680 (*mSin3A N680*), 479 (*mSin3A 479*), or 205 (*mSin3A N205*) were passed over GST columns. Columns contained either plain GST, GST fused to amino acids 32–122 of REST (REST), or GST fused to amino acids 379–928 of retinoblastoma protein (*Rb*). Shown is an autoradiogram of bound proteins after elution from GST columns and separation by SDS-polyacrylamide gel electrophoresis. The arrows indicate the positions of migration of each of the mSin3A constructs. *B*, schematic representation of mSin3A constructs used in the GST pull-down experiments. *Black filled boxes* represent the PAH domains of mSin3A. The ability of each mSin3A constructs to bind GST-REST-(32–122) is indicated as + or −.

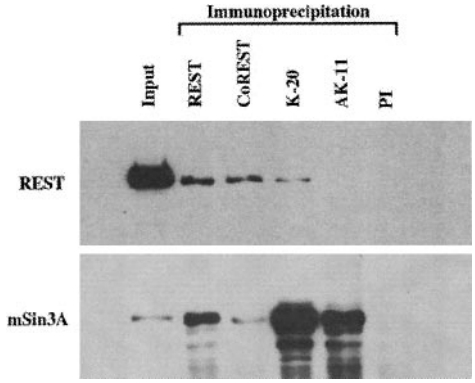

FIG. 3. **REST is present in protein complexes precipitated by antibodies to CoREST and mSin3A in mammalian cells.** Nuclear extracts from REST-FLAG-transfected HEK-293 cells were immunoprecipitated with antibodies directed against REST, CoREST, the NH_2 terminus of mSin3A (*K-20*), the PAH-2 domain of mSin3A (*AK-11*), or preimmune rabbit IgGs (*PI*). The presence of REST and mSin3A in immunoprecipitates and in 10 μg of the input nuclear extract was examined following SDS-polyacrylamide gel electrophoresis and Western blotting using anti-FLAG and anti-mSin3A antibodies. The migration positions of REST (200 kDa) and mSin3A (150 kDa) are indicated.

Sections were hybridized overnight at 50 °C in hybridization buffer (0.6 M NaCl, 10 mM Tris-Cl, pH 7.5, 1× Denhardt's reagent, 1 mM EDTA, 0.01% sheared herring sperm DNA, 0.05% yeast total RNA, 0.005% yeast tRNA, 50% formamide, and 10% dextran sulfate) containing a minimum of 0.4 ng/μl/kilobase of cRNA probe. Hybridization signals were detected using an alkaline phosphatase-labeled antibody to digoxygenin and visualized with the chromogen nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

The Co-repressor mSin3A Interacts with a Domain in REST Required for Repression—In a yeast two-hybrid screen in which mSin3A was used as the bait, three cDNAs out of a total of 20 sequenced represented REST fragments. Two cDNAs encoded amino acids 32–122 of REST, and one cDNA encoded amino acids 8–135. To test whether the mSin3A interaction was specific for the NH_2 terminus of REST, two LexA-REST fusion proteins were generated, one encoding amino acids 1–525 (N-REST), and the other encoding amino acids 525–1097 (C-REST). The pGAD-mSin3A proteins interacted only with N-REST (Fig. 1). Unfortunately, LexA-full-length REST was not stable in yeast, and therefore we were unable to demonstrate interaction of full-length REST with mSin3A using this system. The mSin3A-interacting domain of REST overlapped with a domain that was shown previously to be required and sufficient for repression of type II sodium channel reporter genes (7). To determine whether mSin3A might also interact with the co-repressor that mediates repression from the COOH-terminal repressor domain of REST, CoREST, further two hybrid analyses were performed (Fig. 1). No interactions were observed between mSin3A or an NH_2-terminal fragment of mSin3A, with either full-length CoREST (Fig. 1) or NH_2-terminal (amino acids 1–293) and COOH-terminal (amino acids 101–482) fragments of CoREST (data not shown).

The mSin3A co-repressor contains four domains that mediate protein-protein interactions, and three of the domains (PAH1–3) are clustered at the NH_2 terminus of the protein. A yeast interaction assay using pGAD N-mSin3A showed that the interaction of REST was also through the NH_2 terminus of mSin3A (Fig. 1). To further characterize the interaction, an *in vitro* binding assay was performed. A GST fusion protein containing amino acids 32–122 of REST was expressed in bacteria and then purified and immobilized on glutathione-Sepharose beads. *In vitro* translated mSin3A protein, labeled with [^{35}S]methionine, bound to a GST-retinoblastoma protein col-

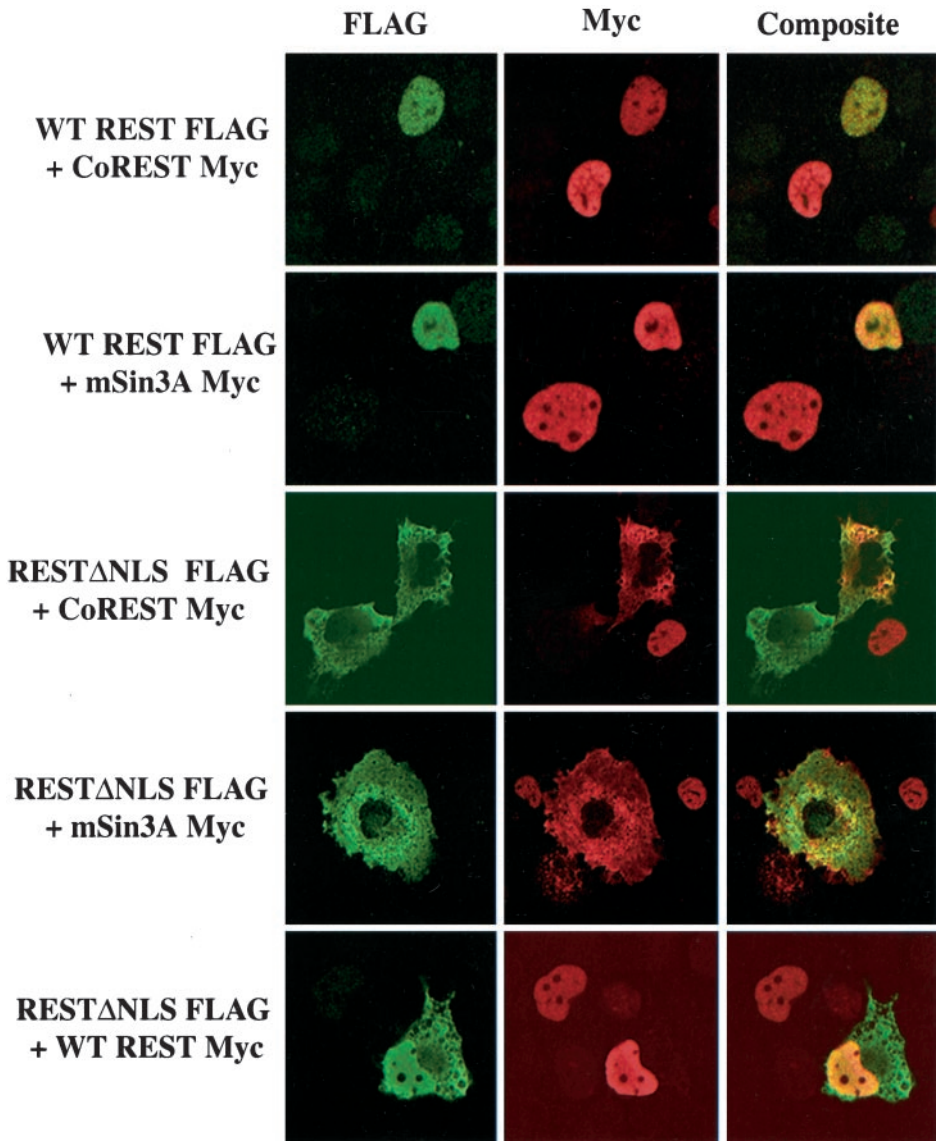

FIG. 4. **Co-localization of CoREST and mSin3A with a cytoplasmic REST mutant in intact mammalian cells.** COS-1 cells were co-transfected with combinations of expression constructs encoding FLAG-tagged full-length REST (*WT REST FLAG*), a FLAG-tagged mutant form of REST from which the consensus nuclear localization signal had been deleted (*REST ΔNLS FLAG*), Myc-tagged full-length REST (*WT REST Myc*), Myc-tagged CoREST (*CoREST Myc*), and Myc-tagged mSin3A (*mSin3A Myc*), as indicated. After fixation and double label immunostaining, cells were examined by confocal immunofluorescence microscopy. *Green* and *red* staining represent FLAG and Myc epitopes, respectively. The *far right panel* shows the *red* and *green* images superimposed to give a composite in which *yellow* represents co-localization of FLAG and Myc staining. All images represent 1-μm optical sections.

umn used as a positive control (16). The radiolabeled mSin3A protein also bound to a GST-REST-(32–122) column but not to GST alone (Fig. 2). Analysis of binding of truncated mSin3A products showed that REST interacts with amino acids 205–479 of mSin3A, corresponding to a region containing the paired amphipathic helix domain 2 (PAH-2; Fig. 2).

CoREST and mSin3A Form a Complex with REST in Mammalian Cells—The interactions between REST and mSin3A observed in the yeast two-hybrid assay and *in vitro* were confirmed in mammalian cells using two approaches. First, co-immunoprecipitation experiments were performed using nuclear extracts from HEK-293 cells overexpressing REST-FLAG under control of a tetracycline-inducible promoter. FLAG epitopes were present in immunoprecipitates using REST, CoREST, and mSin3A K-20 antibodies but not in immunoprecipitates using mSin3A AK-11 antibody or preimmune IgG (Fig. 3). Conversely, mSin3A was present in immunoprecipitates using REST, CoREST, and mSin3A antibodies (both K-20 and AK-11; Fig. 3). Given that the AK-11 antibodies recognize the PAH-2 domain in mSin3A, while the K-20 antibodies recognize epitopes in the mSin3A amino terminus, these results are consistent with the GST pull-down experiments above indicating REST interaction through the mSin3A PAH-2 domain.

Second, REST interacted with CoREST and mSin3A overexpressed in intact mammalian cells. In these studies, an epitope-tagged version of REST cDNA lacking a nuclear localization signal (RESTΔNLS-FLAG) was transfected into COS-1 cells. In contrast to WT REST, which is restricted to the nucleus, RESTΔNLS-FLAG was predominantly cytoplasmic, as visualized by immunocytochemistry using an anti-FLAG antibody (Fig. 4). The mutated REST protein caused a dramatic redistribution of both CoREST- and mSin3A-myc fusion proteins to the cytoplasm, whereas they were both strictly nuclear in the presence of WT REST (Fig. 4). Quantitation of the data indicated that mSin3A-myc was redirected to the cytoplasm in 98% of cells expressing RESTΔNLS-FLAG (n = 50). Furthermore, the proteins appeared to co-localize within aggregates in the cytoplasm, indicated by the superimposition of the fluorescence

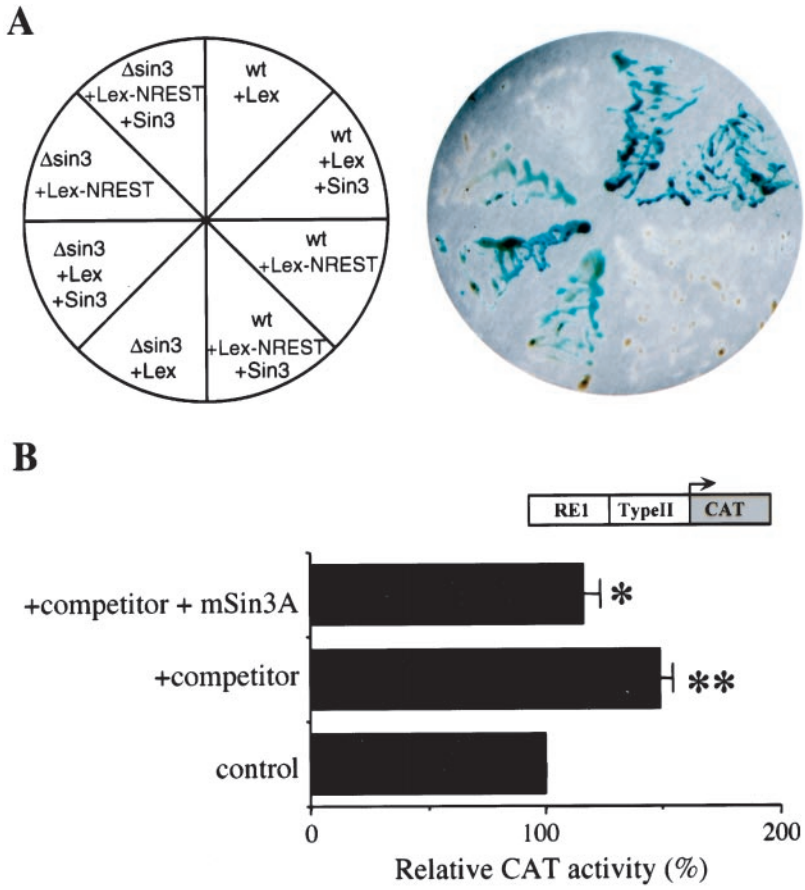

FIG. 5. **mSin3A is required for the repressor function of the NH_2-terminal domain of REST.** *A*, yeast WT and Sin3 deletion strains were cotransformed with the indicated plasmids and plated for assaying β-galactosidase activity derived from an integrated *LexA-LacZ* reporter. Two separate sets of transformations yielded identical results. *B*, graph of relative CAT activity following co-transfection of HEK293 with a type II sodium channel CAT reporter gene, a "competitor" peptide containing the NH_2-terminal repressor domain of REST, and mSin3A, as indicated. The level of CAT activity resulting from expression of the CAT reporter gene alone was set to 100%. The graph represents the mean data from six experiments, and *bars* represent the S.E. The statistical significance of differences between the data sets was determined using the nonparametric Mann-Whitney *U* test. *, $p = 0.001$ for experiment with competitor compared with the no competitor control. **, $p = 0.008$ for competitor alone compared with competitor plus mSin3A.

(Fig. 4). Similarly, in 96% of cells expressing RESTΔNLS-FLAG in the cytoplasm, CoREST-myc was found in the cytoplasm ($n = 50$). To eliminate the possibility that there was a fortuitous interaction of the epitopes or that overexpression of the proteins *per se* led to coincidental co-localization in the cytoplasm, RESTΔNLS-FLAG was co-transfected with cDNA coding for WT REST-myc. In this case, in 100% of the cells that showed cytoplasmic FLAG staining, REST-myc immunostaining remained strictly nuclear ($n = 50$).

mSin3A Is a Functional Co-repressor for REST—The finding that mSin3A binds to REST through its NH_2-terminal repressor domain suggested to us that mSin3A could function as a co-repressor for REST. To test this idea, functional experiments were performed in both yeast and mammalian cells. In a WT yeast strain, expression of the NH_2 terminus of REST fused to the LexA DNA binding domain (LexA N-REST) resulted in complete repression of a LexA-βGal reporter gene, whereas LexA N-REST was unable to repress reporter gene expression in a Sin3 minus yeast strain (Fig. 5*A*). Furthermore, this lack of repressor activity of LexA N-REST in the Sin3 null strain could be rescued by co-expression of Sin3 (Fig. 5*A*).

To test whether mSin3A is involved in repression by REST in mammalian cells, a competition assay was performed using a peptide from REST containing the mSin3A interaction domain (Gal4F4). HEK293 cells were transfected with a RE1 type II sodium channel CAT reporter gene, pSDK7. This reporter construct is expressed at low levels due to its repression by the endogenous REST protein (Fig. 5*B*). In the presence of the competitor peptide, the type II sodium channel reporter gene was derepressed, presumably due to binding of endogenous mSin3A to the competitor peptide. (Fig. 5*B*). In the absence of competitor peptide, overexpression of mSin3A has no effect on activity of the reporter gene (data not shown).

mSin3A and CoREST Are Differentially Localized during Development—An *in situ* hybridization study was performed using antisense riboprobes specific for mouse mSin3A, REST, and CoREST. Sense riboprobes were used as controls in each situation and resulted in no obvious staining (data not shown). At embryonic day 8.5, both the REST and mSin3A genes were expressed widely throughout the embryo, including the somites, the presomitic mesoderm, and the head mesenchyme (Fig. 6). However, at this stage, CoREST exhibited a much more restricted pattern of expression, with strong expression in the head mesenchyme, but no visible staining in either the somites or the presomitic mesoderm (Fig. 6). By day 11.5 of development, the disparity between the expression patterns of the two co-repressors was no longer apparent. Both mSin3A and CoREST appeared to be expressed fairly ubiquitously throughout the embryo (Fig. 6).

DISCUSSION

The repressor protein REST/NRSF plays a crucial role in suppressing the neuronal phenotype outside of the developing nervous system. However, until recently, little was known about the mechanism by which REST repressed its target genes. A previous study determined that a newly identified co-repressor, CoREST, mediated repression from the COOH terminus of REST (1). In this study, we investigated whether the known co-repressor mSin3A, a crucial component of many inducible repression systems, might also be involved in repression by REST. We found, using several approaches, that mSin3A interacts with the NH_2-terminal repressor domain REST and that this interaction is required for function.

Co-immunoprecipitation studies revealed that REST is present in a complex with both mSin3A and CoREST in nuclei isolated from HEK293 cells. By exploiting the finding that removal of a consensus NLS in REST relocalized it to the cytoplasm, we were also able to visualize the interaction of

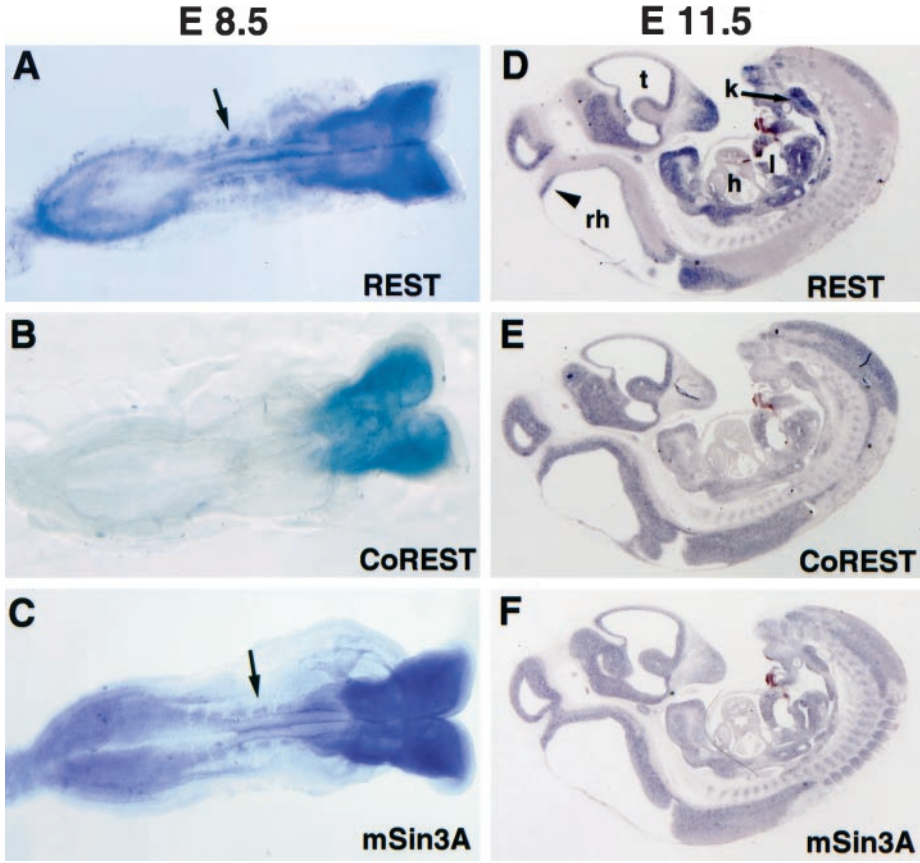

FIG. 6. **mSin3A and CoREST are differentially localized during mouse development.** *A–C* show cRNA hybridization of REST, CoREST, and mSin3A in whole mount day 8.5 mice (*E 8.5*) with 5–7 somites. REST binding (*A*) is localized to the head mesenchyme, the fully formed somites, and the lateral mesenchyme. CoREST binding (*B*) is primarily in the head mesenchyme with no binding seen in the somites or other tissue below the head. Not visible in this plane of view is the positive binding to the primitive heart. mSin3A binding (*C*) is essentially the same as that seen for REST with the exception of the density in the presomitic lateral mesenchyme condensations. Unlike CoREST, neither REST nor mSin3A show binding to the primitive heart. The *arrow* indicates somitic binding. *D–F* show adjacent serial paraffin sections from an 11.5-day mouse embryo (*E 11.5*), hybridized with cRNA probes for REST, CoREST, and mSin3A. REST binding (*D*) is localized to the ventricular germinal layer of the neural tube (*arrowheads*), liver, branchial arch, and primordial kidney. Small bands of binding are also visible in myotome structures. No binding is seen in the intermediate and marginal zones or in the dorsal root ganglia. In addition, no binding is seen in the heart (*D*). CoREST (*E*) and mSin3A (*F*) are expressed in all tissues in which REST is expressed, and in addition they are expressed in all layers of the neural tube, the dorsal root ganglia, and the heart. *h*, heart; *k*, kidney; *l*, liver; *rh*, rhombencephalic vesicle; *t*, telencephalic vesicle.

REST with mSin3A and CoREST in intact mammalian cells, thus circumventing the potential problem of disruption of complexes during cell extraction procedures. The observation that REST, mSin3A, and CoREST are capable of associating in the cytoplasm indicated that interaction of the co-repressors with REST did not require REST to be bound to its target DNA sequence.

REST and mSin3A were also found to interact in an *in vitro* "GST pull-down" assay. Furthermore, the combination of the GST pull-down and yeast two-hybrid experiments mapped the sites of interaction on the two proteins. Amino acids 32–122 of REST were sufficient for interaction with mSin3A. Importantly, this 90-amino acid region overlaps with the NH_2-terminal domain of REST (amino acids 1–83) previously shown to be required for its repressor activity (7), suggesting that binding of mSin3A is responsible for the repressor activity of this domain. The mSin3A-interacting domain of REST mapped to the region of mSin3A containing PAH-2 and flanking sequences. This was confirmed by the co-immunoprecipitation studies. At present, it is not clear whether the binding of REST to mSin3A is direct or whether it involves other bridging proteins that may be present in the reticulocyte lysate used for the *in vitro* translation of mSin3A products.

The site on mSin3A through which REST interacts is involved in binding of another DNA-binding protein, Mad (13, 17), and partially overlaps with the site of interaction of the co-repressors SMRT (18, 19) and NCoR (20) and the general transcription factor TFIIB (18). Interestingly, alternatively spliced forms of mSin3B, containing only PAH-1 and PAH-2 and lacking the histone deacetylase interaction domain, have been identified (21). It has been proposed that these shorter forms of mSin3B may attenuate repressor function. Therefore, since REST also appears to bind to PAH-2, it is possible that repression by REST could also be modulated by interaction with shorter dominant negative forms of mSin3. This could provide a way of dynamically regulating the REST repressor complex.

The NH_2-terminal repressor domain of REST requires mSin3A for repression. This was most strikingly apparent in yeast, where the ability of the NH_2 terminus of REST to repress was abolished in a Sin3-negative yeast strain. In mammalian cells, the ability of endogenous REST to repress reporter genes could be decreased by overexpression of the NH_2-terminal repressor domain of REST, and this effect could be reversed by co-expression of mSin3A. Although significant, these effects were relatively small, probably due to the presence the COOH-terminal repression domain of REST.

It has been shown that mSin3A/B interacts with sequence-specific DNA-binding proteins to recruit multiprotein complexes containing histone deacetylases, resulting in the local-

ized deacetylation of histones in the region around specific promoters (22). This is believed to cause nucleosomes to take on a highly condensed structure, preventing access of the transcriptional activation machinery and resulting in transcriptional repression (23, 24). However, it is known that Sin3 proteins are able to mediate repression even in the absence of histone deacetylase activity, suggesting that mSin3A/B utilizes multiple mechanisms for repression (18, 25). In fact, mSin3A is able to bind the general transcription factor TFIIB, suggesting that mSin3A may also repress through direct inhibition of transcriptional initiation (18). Thus, mSin3A-mediated repression through the NH_2-terminal repression domain of REST could involve histone deacetylation as reported recently (26) and/or direct inhibition of the transcriptional machinery. Unlike the case for the NCoR and SMRT co-repressors, CoREST did not appear to bind to mSin3A. It remains to be determined whether the two co-repressors utilized by REST converge on components of a common repressor complex or whether the two function by completely independent mechanisms.

REST may utilize mSin3A and CoREST differentially during development. *In situ* hybridization data showed that at day 8.5 of mouse development mSin3A was expressed fairly ubiquitously, whereas CoREST expression was restricted only to the head region. After this time, by day 11.5, the expression patterns of mSin3A and CoREST were overlapping and extended to most tissues in the embryo. This result suggests either that REST can repress its target genes through recruitment of mSin3A in the absence of a COOH-terminal co-repressor or that other co-repressors can substitute for CoREST at this stage of development. REST repressor complexes may function differently depending on whether they utilize mSin3A alone or in combination with other co-repressors. Future studies to distinguish between these possibilities will provide insight into the processes by which the neuronal phenotype is established and maintained during development.

Acknowledgments—We are most grateful to Janet Allopenna for technical assistance and Nurit Ballas for critical reading of the manuscript. We are also grateful to David Stillman, Carol Laherty, Bob Eisenman, and Rolf Sternglanz for materials donated.

REFERENCES

1. Andres, M. E., Burger, C., Peral-Rubio, M. J., Battaglioli, E., Anderson, M. E., Grimes, J., Dallmanm J., Ballas, N., and Mandel, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9873–9878
2. Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuller, Y. M., Frohmann, M. A., Kraner, S. D., and Mandel, G. (1995) *Cell* **80,** 949–957
3. Schoenherr, C. J., and Anderson., D. J. (1995) *Science* **267,** 1360–1363
4. Schoenherr, C. J., Paquette, A. J., and Anderson, D. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9881–9886
5. Chen, Z. F., Paquette, A. J., and Anderson, D. J. (1998) *Nat. Genet.* **20,** 136–142
6. Kallunki, P., Edelman, G. M., and Jones, F. S. (1997) *J. Cell Biol.* **138,** 1343–1354
7. Tapia-Ramirez, J., Eggen, B. J. L., Peral-Rubio, M. J., Toledo-Aral, J. J., and Mandel, G. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 1177–1182
8. Leichter, M., and Thiel, G. (1999) *Eur. J. Neurosci.* **11,** 1937–1946
9. Kraner, S. D., Chong, J. A., Tsay, H. J., and Mandel, G. (1992) *Neuron* **9,** 37–44
10. Lavender, P., Vandel., L., Bannister, A. J., and Kouzarides, T. (1997) *Oncogene* **14,** 2721–2728
11. Bannister, A. J., Cook, A., and Kouzarides, T. (1991) *Oncogene* **6,** 1243–1250
12. Wang, H., and Stillman, D. J. (1993) *Mol. Cell. Biol.* **13,** 1805–1814
13. Kasten, M. M., Ayer, D. E., and Stillman, D. J. (1996) *Mol. Cell. Biol.* **16,** 4215–4221
14. Wilkinsin, D. G. (1992) *In Situ Hybridization: A Practical Approach*, IRL Press, Oxford
15. Hockfield, S., Carlson, S., Evans, C., Levitt, P., Pintar, J., and Silberstein, L. (1993) *Molecular Probes of the Nervous System*, Vol. 1, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Tokitou, F., Nomura, T., Khan, M. M., Kaul, S. C., Wadhwa, R., Yasukawa, T., Khono, I., and Ishii, S. (1999) *J. Biol. Chem.* **274,** 4485–4488
17. Ayer, D. E., Lawrence, Q. A., and Eisenman, R. N. (1995) *Cell* **80,** 767–776
18. Wong, C. W., and Privalsky, M. L. (1998) *Mol. Cell. Biol.* **18,** 5500–5510
19. Nagy, L., Kao, H. Y., Chakravarti, D., Lin, R. J., Hassig, C. A., Ayer, D. E., Schreiber, S. L., and Evans, R. M. (1997) *Cell* **89,** 373–380
20. Heinzel, T., Lavinsky, R. M., Mullen, T. M., Soderstrom, M., Laherty, C. D., Torchia, J., Yang, W. M., Brard, G., Ngo, S. D., Davie, J. R., Seto, E., Eisenman, R. N., Rose, D. W., Glass, C. K., and Rosenfeld, M. G. (1997) *Nature* **387,** 43–48
21. Alland, L., Muhle, R., Hou, H., Potes, J., Chin, L., Schreiber-Agus, N., and DePinho, R. A. (1997) *Nature* **387,** 49–55
22. Kadosh, D., and Struhl, K. (1998) *Mol. Cell. Biol.* **18,** 5121–5127
23. Pazin, M. J., and Kadonaga, J. T. (1997) *Cell* **89,** 325–328
24. Wolffe, A. P. (1997) *Nature* **387,** 16–17
25. Laherty, C. D., Yang, W. M., Sun, J. M., Davie, J. R., Seto, E., and Eisenman, R. N. (1997) *Mol. Endocrinol.* **11,** 682–692
26. Huang, Y., Myers, S. J., and Dingledine, R. (1999) *Nat. Neurosci.* **10,** 867–872