Biochemical and Biophysical Research Communications **290,** 415–420 (2002)
doi:10.1006/bbrc.2001.6194, available online at http://www.idealibrary.com on IDEAL®

REST4-Mediated Modulation of REST/NRSF-Silencing Function during BDNF Gene Promoter Activation

Akiko Tabuchi,*,† Tomoko Yamada,* Shoko Sasagawa,* Yoshihisa Naruse,‡ Nozomu Mori,‡ and Masaaki Tsuda*,†,[1]

**Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, Sugitani 2630, Toyama 930-0194, Japan; ‡Department of Molecular Genetics, National Institute of Longevity Science, Japan, Oobu, Aichi 474-5822, Japan; and †Core Research of Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Shibuya 3-13-11, Tokyo 150-0002, Japan*

Received November 11, 2001

Neural-restrictive silencer element (NRSE)/repressor element-1 (RE1) regulates neuron-specific gene expression by binding the transcriptional factor REST/NRSF which functions as a silencer in nonneuronal cells. In neuronal cells, a truncated, neuronal-specific REST/NRSF isoform, REST4, has been found but little is known about its function. To address this, we investigated the effect of REST/NRSF and REST4 on the activity-dependent activation of BDNF gene promoter I (BDNFp-I) using cultured rat cortical neurons. REST/NRSF markedly repressed the transcriptional activation of BDNFp-I, whereas the effect of REST4 was weak, depending upon the NRSE/RE1 sequence. In addition, REST4 enhanced the basal transcriptional activity of BDNFp-I. Coexpression of REST4 with REST/NRSF competitively inhibited the silencing effect of REST/NRSF on the activation of BDNFp-I. Although REST4 itself has a weak repressive effect on activation of the BDNF gene via NRSE/RE1, it can compete the silencing effect of REST/NRSF, suggesting a primary role for REST4 in preventing the neuron-specific gene from being inactivated by REST/NRSF and allowing gene activation in response to a variety of neuronal stimuli.

Key Words: **NRSE/RE1; REST/NRSF; REST4; BDNF; neuronal activity, cortical neuron; calcium.**

Neuronal-restrictive silencer element (NRSE)/repressor element-1 (RE1) is a 21-base-pair sequence found in a number of neuron-specific genes including the SCG10 (1), type II sodium channel (2), *N*-methyl-D-aspartate (NMDA) receptor (3), choline acetyltransferase (4) and brain-derived neurotrophic factor (BDNF) genes (5). The zinc finger transcription factor that binds to NRSE/RE1 is known as RE1-silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) (6, 7). Since REST/NRSF can act as a silencer of neuron-specific gene expression in nonneuronal tissues and in undifferentiated neuronal progenitor cells (6, 7), downregulation of REST/NRSF was thought to be required for the acquisition of terminally differentiated phenotypes of neurons. Recently, it was demonstrated that a small amount of full-length REST/NRSF protein was apparently present in neurons and, furthermore, a C-terminally truncated form of NRSF/REST, termed REST4, was specifically expressed in neurons (8, 9). REST4 has five zinc fingers, but its homo-oligomer lacks DNA-binding ability (10). This truncated form is suggested to be induced by neuronal activity via kainate-induced seizure and to act as a negative modulator against REST/NRSF rather than a transcriptional repressor of neuronal genes (8). Furthermore, it was demonstrated that REST4 can form a hetero-oligomer with full-length REST/NRSF, which inhibits the silencing activity of REST/NRSF in a dominant negative manner (11). At present, however, it is still controversial whether REST4 acts as a transcriptional repressor through a weak DNA-binding to NRSE/RE1 (8) or as a negative modulator through an interaction with REST/NRSF (11).

The functions of REST/NRSF and REST4 have thus far been analyzed using established cell lines like PC12 cell. To elucidate the physiological functions of these proteins in neurons, we used a primary culture of rat cortical neuronal cells (12). Our previous report on the activity of NRSE-linked BDNF or the c-*fos* promoter revealed a marked repressive effect of NRSE/RE1 on BDNF promoter I and c-*fos* promoter activation in primary glial cells, but not in primary cortical neurons (12). Furthermore, the overexpression of REST/NRSF completely blocked the activation of BDNFp-I induced by the calcium (Ca^{2+}) signals evoked via L-type

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-76-434-5048. E-mail: tsuda@ms.toyama-mpu.ac.jp.

0006-291X/02 $35.00

voltage-dependent Ca^{2+} channels (L-VDCC) through the NRSE/RE1 sequence. These findings support the usefulness of this reporter gene assay system in the primary culture of rat cortical neurons for elucidating the function of a series of REST/NRSF variants in neurons.

So far, however, the effect of REST4 on gene activation in neurons has never been investigated. To clarify the function of REST4, in this study, we overexpressed REST4 in primary cultured rat cortical neurons and, in particular, investigated whether or not REST4 could affect the membrane depolarization-induced activation of BDNF promoter I through NRSE/RE1 sequence and modulate the silencing function of full-length REST/NRSF in neurons.

MATERIALS AND METHODS

Plasmids and expression vectors. The construction of GL3-SIL(++)-BDNFpI (termed SIL(++)BDNFpI), which carries a duplicated NRSE upstream of BDNF promoter I, and the mutated NRSE plasmid, pGL3-Sm(++)-BDNFp (termed Sm(++)BDNFpI), were described previously (Fig. 1A and Ref. 12). The NRSEs used in this study were derived from the SCG10 gene promoter (1) and two bases (GG), conserved in all NRSE/RE1, were changed to TT for constructing the mutated NRSEs. The myc epitope-tagged expression vectors, pcDNA3-REST/NRSFmyc and pcDNA3-REST4/NRnVmyc, were constructed by the ligation of human REST/NRSF and REST4/NRnV cDNA between the *EcoR*I and the *Xho*I sites of pcDNA 3.1/Myc-His A (Invitrogen), respectively, and the DNA sequences were confirmed by conventional sequencing using an ABI-PE 377 sequencer (Applied Biosystems). Then, they were used for the expression of REST/NRSF and REST4. The biological characteristics such as the subcellular localization or DNA-binding ability of their myc-tagged proteins were consistent with those of wild type REST/NRSF and REST4 (data not shown).

Cell culture. Primary cultures of cortical neurons were prepared from the cerebral cortexes of 17- or 18-day-old rat (CRJ: SD) embryos as described (13). Briefly, small pieces of cerebral cortex were treated with 0.125% trypsin (DIFCO) and 1 mM EDTA for 15 min at 37°C and thoroughly dissociated in Dulbecco's modified eagle medium (DMEM). After centrifugation, the cell pellet was treated with 0.004% DNase I (Sigma) and 0.03% trypsin inhibitor (Sigma) and the suspended cells were seeded at 5×10^6 cells in a 60-mm culture dish (Iwaki). The cells were grown for 48 h in DMEM medium containing 10% fetal calf serum, then the medium was replaced with serum-free DMEM containing glucose (4.5 mg/ml), transferrin (5 μg/ml), insulin (5 μg/ml), sodium selenite (5 ng/ml), bovine serum albumin (1 mg/ml) and kanamycin sulfate (100 μg/ml) (TIS medium). To avoid the proliferation of nonneuronal cells, cytosine arabinoside (Sigma) was also added at 2 μM.

DNA transfection and luciferase assay. DNA transfection of cortical neurons was carried out at 3 DIV after culture, and the procedure was performed as described (14). As an internal control vector, we used pRL-EF1α carrying the human elongation factor 1α promoter upstream of the *Renilla* luciferase gene. The calcium phosphate/DNA precipitates were prepared with 3 μg of luciferase reporter plasmid DNA (pGL3-SIL(++)-BDNFpI/pRL-EF1α or pGL3-Sm(++)-BDNFpI/pRL-EF1α = 25) plus 5 μg of expression vector (pcDNA3-REST/NRSFmyc, pcDNA3-REST4/NRnVmyc or pcDNA 3.1/Myc-His A as an empty vector (Invitrogen)). The cell lysates for luciferase assay were prepared 12 h after treatment with 25 mM KCl. The procedure for the dual luciferase assay was provided by the supplier (Promega).

RESULTS AND DISCUSSION

Repression of BDNF Promoter Activity by REST4

We have already reported that the silencing effect of NRSE was observed only in nonneuronal cells, and not in primary cortical neurons when a duplicated NRSE was linked upstream of BDNF-pI (termed SIL(++)BDNFpI), as illustrated in Fig. 1A (12). REST/NRSF, as shown in Fig. 1B, has 9 zinc finger motifs, whereas REST4 has only 5, which were identical to the first five zinc finger motifs of full-length REST/NRSF. To investigate whether REST/NRSF and REST4 affect the membrane depolarization-induced SIL(++)BDNFpI activation, we co-transfected SIL(++)BDNFpI as a reporter vector, with the vector expressing REST/NRSF or REST4, into primary cultured rat cortical neurons. As shown in Fig. 2A, the increase of transcription driven by SIL(++)BDNFpI was observed on stimulation with 25 mM KCl in the absence of REST/NRSF, but the coexpression of REST/NRSF strongly suppressed this increase even at 1 μg of REST/NRSF vector plasmid, which was consistent with the previous report (12). In contrast, the coexpression of REST4 allowed the transcriptional activation but a slight, dose-dependent reduction of activation was observed (Fig. 2B, see the columns at +KCl). When 25 mM KCl was not administered, the co-expression of REST4 slightly increased the basal transcription (Fig. 2B, see the columns at −KCl).

NRSE-Mediated Effect of REST4

We next examined whether the effect of REST4 on the transcriptional activation of BDNFp-I was mediated by the *cis*-element, NRSE/RE1, or not. When NRSE-free (BDNFpI), NRSE-linked (SIL(++)BDNFpI) and mutated NRSE-linked BDNF promoter I (Sm(++)BDNFpI) were transfected only with empty vector plasmid, transcriptional activation induced by KCl-treatment was commonly observed (Figs. 3A and 3B). Under the expression of REST/NRSF, however, repression of the transcriptional activation was observed with SIL(++)BDNFpI but not with BDNFpI and Sm(++)BDNFpI (Fig. 3A, see the columns at +KCl). These findings indicate that the silencing effect of REST/NRSF on the transcriptional activation of BDNFp-I is mediated by NRSE, which is consistent with the previous report (12). On the other hand, a weak silencing effect was detected using SIL(++)BDNFpI but not BDNFpI or Sm(++)BDNFpI when REST4 was expressed (Fig. 3B), indicating that NRSE was also required for the weak repressive effect of REST4. As was already pointed out in Fig. 2B, furthermore, a slight increase in the basal transcription of SIL(++)BDNFpI was also detected when REST4 was expressed (see the legend to Fig. 3). These findings indicate that the effect not only of REST/

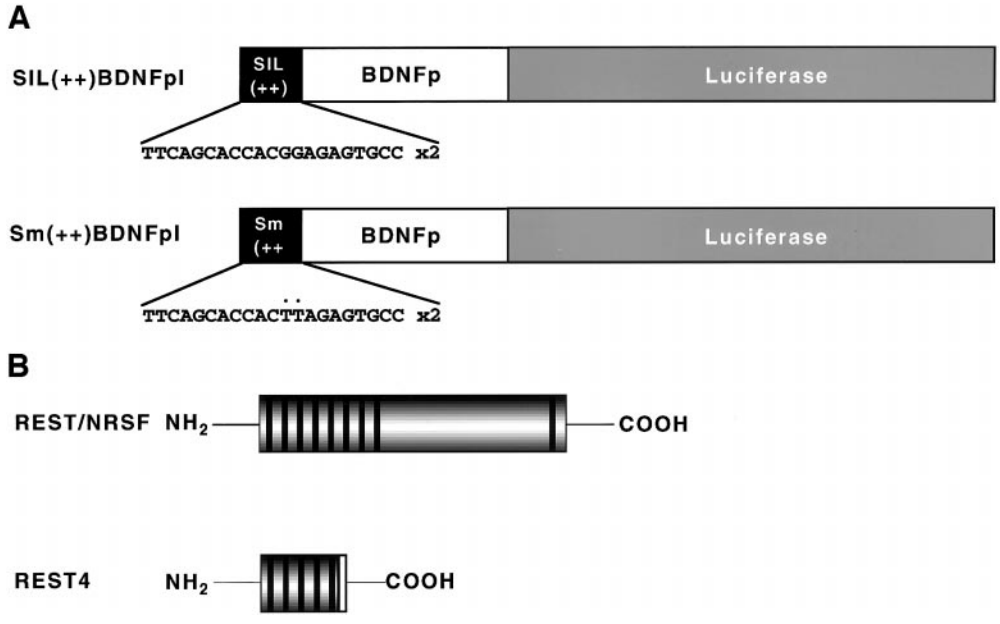

FIG. 1. Reporter vectors and the structure of REST/NRSF and REST4. (A) The structure of SIL(++)BDNFpI and Sm(++)BDNFpI. The two reporter vectors were constructed by the duplicated insertion of SCG10-derived NRSE or its mutated form (the base substitution of GG to TT as shown by dots) upstream of BDNF promoter I. The construction of these vectors was described previously (12). (B) Comparison between full-length and splice variants of REST/NRSF. Full-length REST/NRSF has 8 zinc fingers in the N-terminal portion and one zing finger at the C-terminal end, whereas REST4 has only 5 zinc finger domains that are identical to the corresponding region of REST/NRSF and an additional 12 amino acid residues derived from the neuron-specific exon (8). The zinc finger is represented by a closed square. The neuron-specific exon is represented by an open square.

NRSF but of REST4 on the BDNF gene promoter I activity is mediated by NRSE/RE1.

Competitive Effect of REST4 on the REST/NRSF-Mediated Silencing Effect

It has already been reported that REST4 acts as a dominant negative factor rather than as a transcriptional repressor because it interacts with REST/NRSF (11). Here we examined whether REST4 competes with REST/NRSF for the transcriptional activation of BDNFp-I. As shown in Fig. 4, the expression of REST/NRSF repressed the membrane depolarization-induced activation driven by SIL(++)BDNFpI but the repression was partially abolished when the REST4 was coexpressed with REST/NRSF. This competitive recovery of transcriptional activation increased dependent on the concentration of REST4 vector plasmid. Even though REST4 was expressed in excess on addition of the REST4 vector plasmid at more than 5 μg, the silencing effect of REST/NRSF was not completely abolished (data not shown).

In the present study, we first demonstrated that the effect of the REST/NRSF splice form, REST4, on the transcriptional activation of the NRSE-linked BDNF promoter I was repressive in rat cortical neurons and was mediated by NRSE/RE1 sequence (Figs. 2 and 3). However, the extent of the repression induced by REST4 was weak, compared with the strong repression of REST/NRSF (Fig. 2). Using a gel-mobility assay, Palm *et al.* (8) reported that REST4 had no activity to bind to NRSE/RE1. However, Lee *et al.* (10) recently reported that REST4 can form homo-oligomers but only the monomer binds to NRSE/RE1 at a lower DNA-binding affinity than that of REST/NRSF. Therefore, the weak repression caused by REST4 through NRSE/RE1 sequence might be due to the DNA-binding of the REST4 monomer to NRSE/RE1, which could be brought about by the overexpression of REST4 in the transfected cortical neurons.

Using a gel-mobility assay, on the other hand, Shimojo *et al.* (11) reported that the DNA-binding of REST/NRSF to NRSE/RE1 sequence was competitively inhibited by REST4. Using DNA transfection, in the present study, we clearly demonstrated the competitive interaction between REST/NRSF and REST4 in primary rat cortical neurons, in which REST4 played a dominant negative role as a competitor against the silencing function of full-length REST/NRSF (Fig. 4). It was also reported that the interaction of REST4 with REST/NRSF resulted in the formation of a hetero-oligomer complex, which might prevent the binding of REST/NRSF to NRSE/RE1 as a dominant negative action (11). Although it is still uncertain whether this competitive action of REST4 in neurons is due to a competitive binding of the REST4 monomer to NRSE/RE1 or to a dominant negative action of REST4 via the

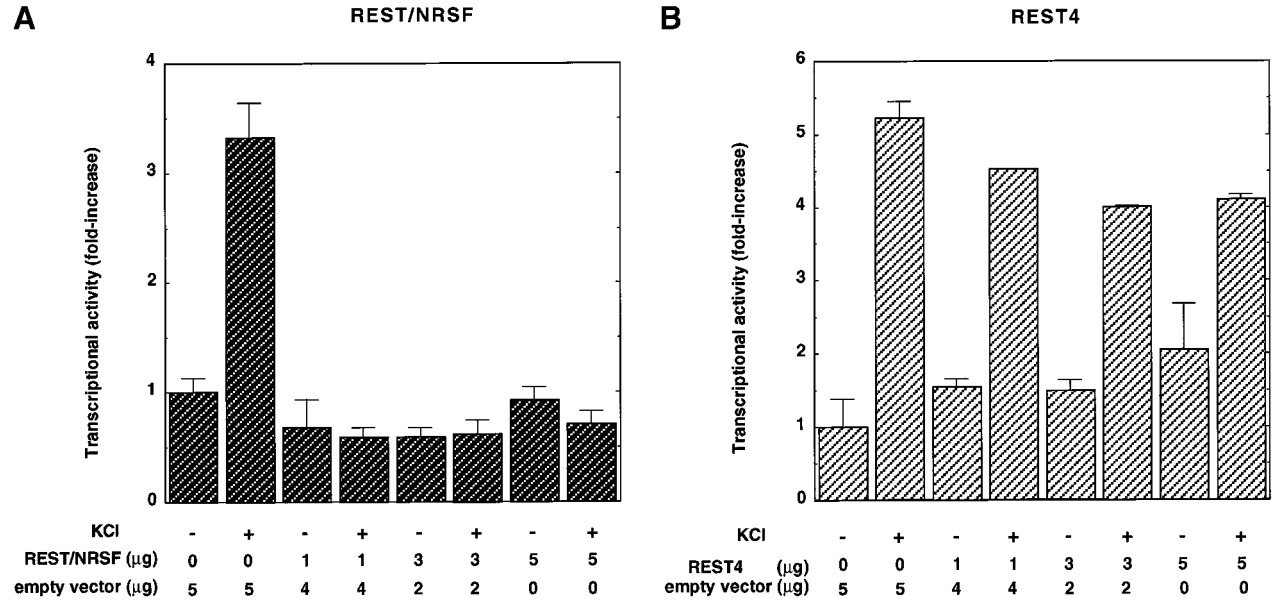

FIG. 2. Repression of depolarization-induced BDNF promoter activation by REST/NRSF and REST4. pcDNA3-REST/NRSFmyc and pcDNA3-REST4/NRnVmyc, which express myc-tagged REST/NRSF (A) and myc-tagged REST4 (B), respectively, were cotransfected with SIL(++)BDNFpI reporter vector into rat cortical neurons 3 days after culture. Cells were stimulated with 25 mM KCl 16 h after transfection, and cell lysates were prepared 12 h later. Total DNA plasmid (5 μg) was an expression vector containing the amounts indicated. Transcriptional activation was indicated as a fold-increase of the control in which REST/NRSF or REST4 was not transfected. Data represent the mean ± SEM (n = 3–4). The same tendency was observed in at least two independent experiments.

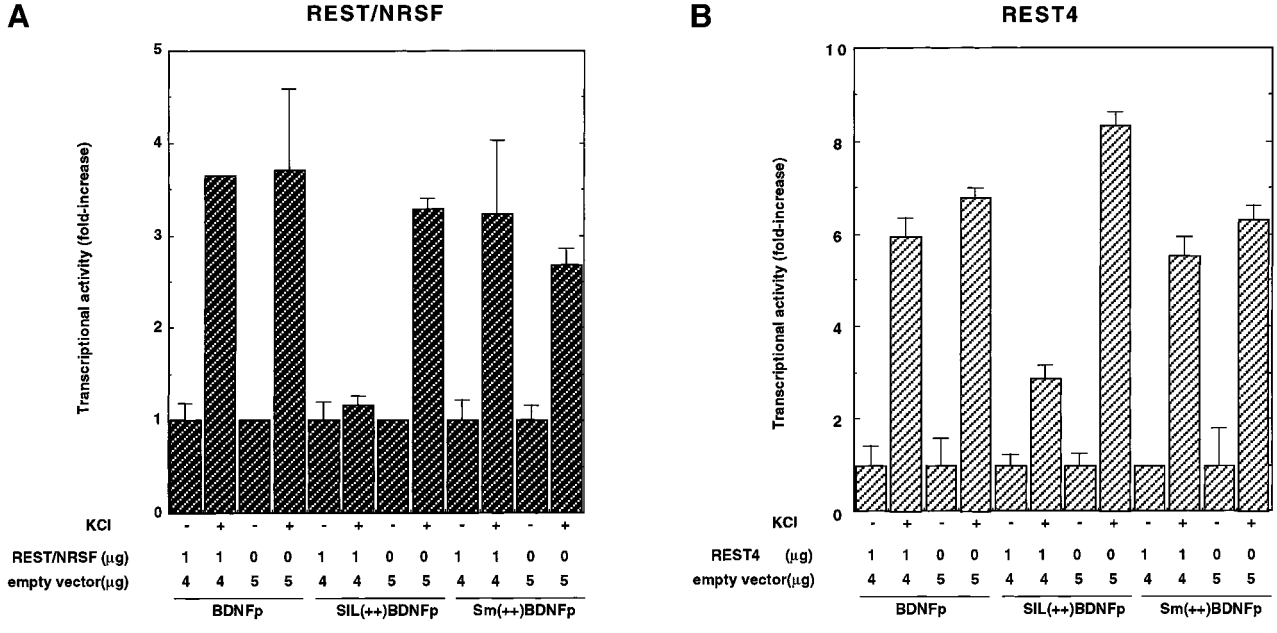

FIG. 3. NRSE-mediated effect of REST/NRSF and REST4. Expression vectors used and the time schedule for DNA transfection and KCl stimulation were as described in the legend to Fig. 2. NRSE-free promoter, BDNFpI, SIL(++)BDNFpI and Sm(++)BDNFpI were transfected as the reporter vectors with myc-tagged REST/NRSF (A) or myc-tagged REST4 (B). Transcriptional activity was indicated as a fold-increase of each control in which the KCl stimulation was omitted. Data represent the mean ± SEM (n = 3–4). The same tendency was observed in at least two independent experiments. The basal transcriptional activities (arbitrary unit) of unstimulated samples (B) were 1.15 (+REST4, +BDNFp), 1.0 (−REST4, +BDNFp), 2.92 (+REST4, +SIL(++)BDNFp), 1.67 (−REST4, +SIL(++)BDNFp), 2.29 (+REST4, +Sm(++)BDNFp) and 1.25 (−REST4, +Sm(++)BDNFp), respectively.

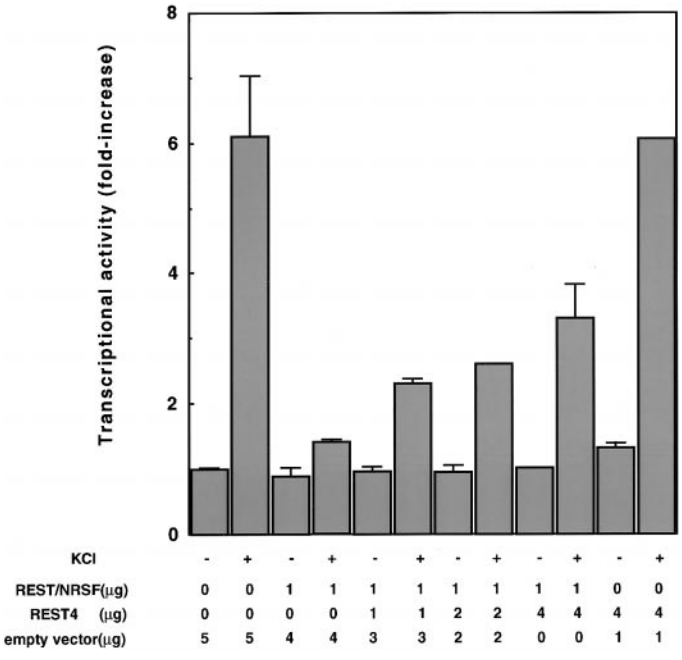

FIG. 4. Competitive inhibition of NRSF-repressive activity by REST4. Expression vectors used and the time schedule for DNA transfection and KCl stimulation were as described in the legend to Fig. 2. The SIL(++)BDNFp was used as a reporter plasmid. Transcriptional activity was indicated as a fold increase of the control in which REST4 and REST/NRSF were not transfected. Data represent the mean ± SEM (n = 3–4). The same tendency was observed in at least two independent experiments.

formation of a hetero-oligomer with REST/NRSF, it seems unlikely that the competitive binding of REST4 to NRSE/RE1 accounts for the competitive action of REST4 (Fig. 4), because the DNA-binding affinity of REST4 to NRSE/RE1 is quite low compared to that of REST/NRSF (8–11). In addition, the fact that the coexpression of REST4 increased the basal transcriptional activity of SIL(++)BDNFpI but not BDNFpI (Fig. 2 and see the legend to Fig. 3) seems to support a dominant negative action of REST4 through NRSE/RE1 sequence. The enhancing effect of REST4 on basal transcription was demonstrated with the choline acetylesterase gene (11). In any case, however, it can be speculated that the expression of REST4 in neurons primarily prevents the neuron-specific gene promoters from being repressed by REST/NRSF, whose expression was slightly detected in neurons (8, 9), allowing the transcriptional activation of these genes in response to a variety of stimuli in neurons.

The REST/NRSF-mediated transcriptional repression is due to the interactions with the transcriptional corepressors, mSin3 and CoREST, which interact with the N-terminus and the C-terminus of REST/NRSF, respectively, to recruit histone deacetylase (HDAC) (15, 16). This raises the possibility that the action of REST4, which is devoid of the binding domain with CoREST, interferes with the interaction between full-length REST/NRSF and HDAC, resulting in a disruption of heterochromatin. The BDNF gene has its own natural NRSE, which is located between exons I and II of the BDNF gene (5). REST4 may play a role in keeping the BDNF gene promoters open by excluding HDAC molecules, allowing the transcriptional activation of the BDNF gene in response to the Ca^{2+} signals evoked in neurons.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in Aid from the Ministry of Education, Science, Sports and Culture, Japan and also in part by Core Research of Evolutional Science and Technology (CREST), Japan Science and Technology, Japan.

REFERENCES

1. Mori, N., Schoenherr, C., Vandenbergh, D. J., and Anderson, D. J. (1992) A common silencer element in the SCG10 and type II Na^+ channel genes binds a factor present in nonneuronal cells but not in neuronal cells. *Neuron* **9,** 45–54.
2. Kraner, S. D., Chong, J. A., Tsay, H.-J., and Mandel, G. (1992) Silencing the type II sodium channel gene: Model for neural-specific gene regulation. *Neuron* **9,** 37–44.
3. Bai, G., Norton, D. D., Prenger, M. S., and Kusiak, J. W. (1998) Single-stranded DNA-binding proteins and neuron-restrictive silencer factor participate in cell-specific transcriptional control of the NMDAR1 gene. *J. Biol. Chem.* **273,** 1086–1091.
4. Mieda, M., Haga, T., and Saffen, D. W. (1997) Expression of the rat m4 muscarinic acetylcholine receptor gene is regulated by the neuron-restrictive silencer element/repressor element 1. *J. Biol. Chem.* **272,** 5854–5860.
5. Timmusk, T., Palm, K., Lendahl, U., and Metsis, M. (1999) Brain-derived neurotrophic factor expression *in vivo* is under the control of neuron-restrictive silencer element. *J. Biol. Chem.* **274,** 1078–1084.
6. Chong, J. A., Tapia-Ramirez, J., Kim, S., Toledo-Aral, J. J., Zheng, Y., Boutros, M. C., Altshuller, Y. M., Frohman, M. A., Kraner, S. D., and Mandel, G. (1995) REST: A mammalian silencer protein that restricts sodium channel gene expression to neurons. *Cell* **80,** 949–957.
7. Schoenherr, C., and Anderson, D. J. (1995) The neuron-restrictive silencer factor (NRSF): A coordinate repressor of multiple neuron-specific genes. *Science* **267,** 1360–1363.
8. Palm, K., Belluardo, N., Metsis, M., and Timmusk T. (1998) Neuronal expression of zinc finger transcription factor REST/NRSF/XBR gene. *J. Neurosci.* **18,** 1280–1296.
9. Kojima, T., Murai, K., Naruse, Y., Takahashi, N., and Mori, N. (2001) Cell-type non-selective transcription of mouse and human genes encoding neural-restrictive silencer factor. *Brain Res. Mol. Brain Res.* **90,** 174–186.
10. Lee, J.-H., Shimojo, M., Chai, Y.-G., and Hersh, L. B. (2000) Studies on the interaction of REST4 with the cholinergic repressor element-1 neuron restrictive silencer element. *Brain Res. Mol. Brain Res.* **80,** 88–98.
11. Shimojo, M., Paquette, A. J., Anderson, D. J., and Hersh, L. B. (1999) Protein kinase A regulates cholinergic gene expression in PC12 cells: REST4 silences the silencing activity of neuron-restrictive silencer factor/REST. *Mol. Cell Biol.* **19,** 6788–6795.
12. Tabuchi A., Nakatani, C., Nakaoka R., Naruse, Y., Kojima, T., Mori, N., and Tsuda, M. (1999) Silencer-mediated repression and non-mediated activation of BDNF and c-*fos* gene promoters in

primary glial or neuronal cells. *Biochem. Biophys. Res. Commun.* **261,** 233–237.

13. Tabuchi, A., Nakaoka, R., Amano, K., Yukimine, M., Andoh, T., Kuraishi, Y., and Tsuda, M. (2000) Differential activation of brain-derived neurotrophic factor gene promoters I and III by Ca^{2+} signals evoked via L-type voltage-dependent and *N*-methyl-D-aspartate receptor Ca^{2+} channels. *J. Biol. Chem.* **275,** 17269–17275.
14. Tabuchi, A., Sano, K., Nakaoka, R., Nakatani, C., and Tsuda, M. (1998) Inducibility of BDNF gene promoter I detected by calcium-phosphate-mediated DNA transfection is confined to neuronal but not to glial cells. *Biochem. Biophys. Res. Commun.* **253,** 818–823.
15. Naruse, Y., Aoki, T., Kojima, T., and Mori, N. (1999) Neural restrictive silencer factor recruits mSin3 and histone deacetylase complex to repress neuron-specific target genes. *Proc. Natl. Acad. Sci. USA* **96,** 13691–13696.
16. Ballas, N., Battaglioli, E., Atouf, F., Andres, M. E., Chenoweth, J., Anderson, M. E., Burger, C., Moniwa, M., Davie, J. R., Bowers, W. J., Federoff, H. J., Rose, D. W., Rosenfeld, M. G., Brehm, P., and Mandel, G. (2001) Regulation of neuronal traits by a novel transcriptional complex. *Neuron* **31,** 353–365.