The zinc finger protein RE-1-silencing transcription factor (REST) is a transcriptional repressor that represses neuronal genes in nonneuronal tissues. Transfection experiments of neuroblastoma cells using a REST expression vector revealed that synapsin I promoter activity is controlled by REST. The biological activity of REST was further investigated using a battery of model promoters containing strong promoters/enhancers and REST binding sites. REST functioned as a transcriptional repressor when REST binding motifs derived from the genes encoding synapsin I, SCG10, α2-glycine receptor, the β2-subunit of the neuronal nicotinic acetylcholine receptor, and the m4-subunit of the muscarinic acetylcholine receptor were present in the promoter region. No differences in the biological activity of these REST binding motifs tested were detected. Moreover, we found that REST functioned very effectively as a transcriptional repressor at a distance. Thus, REST represents a general transcriptional repressor that blocks transcription regardless of the location or orientation of its binding site relative to the enhancer and promoter. This biological activity could also be attributed to isolated domains of REST. Both repressor domains identified at the N and C termini of REST were transferable to a heterologous DNA binding domain and functioned from proximal and distal positions, similar to the REST protein.

RE-1-silencing transcription factor (REST), also known as neuron-restrictive silencer factor, functions as a transcriptional repressor of neuronal genes in nonneuronal tissues (1, 2). Target genes of REST are the genes encoding choline acetyltransferase, the type II sodium channel, SCG10, the m4 muscarinic acetylcholine receptor, and the adhesion proteins L1 and NgCAM (1–6). We have shown recently that the REST binding motif termed the neural-restrictive silencer element (NRSE) was identified at various positions in those genes regulated by REST. NRSEs were found in the promoter region as shown for the synapsin I and the muscarinic acetylcholine receptor m4-subunit genes (7–9). In the SCG10 gene, an NRSE is located farther upstream at position −1472 to −1452 (10), whereas in the NgCAM gene, five NRSEs have been discovered within the first intron (3). Furthermore, genes encoding the α1-glycine receptor and the β2-subunit of the neuronal nicotinic acetylcholine receptor contain NRSEs in the 5′-untranslated region downstream of the transcriptional start site (11, 12). It has been proposed that REST switches its activity from repressing to activating transcription when REST binds to an NRSE located in the 5′-untranslated region or less than 50 base pairs upstream from the TATA box (13).

Most of the NRSEs known so far were detected by homology to the initial NRSEs described in the SCG10 and type II sodium channel gene. Therefore, the biological activity for many NRSEs in recruiting REST and by that means leading to transcriptional repression still has to be shown. A core NRSE has been proposed with the sequence NNCAGCACCNNGCACA-GNNNC, and it was shown that sequences with six or more deviations from the consensus as found in genes encoding the nicotinic acetylcholine receptor α7-subunit, the sodium/potassium ATPase α3-subunit, and dopamine β-hydroxylase did not bind REST (14).

Here, the biological activity of REST was first analyzed on a natural target gene, the synapsin I gene. In addition, the ability of REST to silence (or activate) transcription from proximal and distal positions was investigated with model promoters containing strong promoters/enhancers of viral and mammalian origin. REST binding sites derived from the genes encoding synapsin I, SCG10, α2-glycine receptor, the β2-subunit of the neuronal nicotinic acetylcholine receptor, and the m4-subunit of the muscarinic acetylcholine receptor were inserted at various positions within the transcription unit. Transfection experiments using these reporter constructs together with an expression vector encoding human REST revealed that REST is able to block transcription of a promoter/enhancer from proximal and distal positions. REST functions, therefore, as a silencer-binding protein in a similar, but opposite manner, as an enhancer-binding protein, i.e. REST blocks transcription of a gene when the NRSE is located upstream or downstream of the open reading frame in either orientation and in both a distance- and gene-independent manner.

A transcriptional repressor is typically composed of a DNA- tethering domain, which anchors the protein to DNA, and a repressor domain. This modular structure was investigated to define the DNA binding domain of REST and to elucidate which part of the molecule is involved in transcriptional repression (or activation). DNA binding activity and transcriptional repressor activity could be attributed to different domains.
within the REST protein. We found two repressor domains, one located at the N terminus and the second at the C terminus. Both repressor domains were transferable to a heterologous DNA binding domain of the yeast transcription factor GAL4 and functioned when bound to distal and proximal positions within a gene. Thus, REST and fusion proteins consisting of the REST repressor domains and the DNA binding domain of GAL4 repress transcription by a mechanism that is independent of the position and orientation of the binding site within a transcription unit.

EXPERIMENTAL PROCEDURES

Reporter Constructs—All reporter constructs are derivatives of pOVEC (15). Plasmids pSY400VEC, OVEC-S, and ICPOref have been described (16, 17). Plasmid pgrpOVEC containing the enhancer of the rat grp78 gene was constructed by inserting a 291-nucleotide Small EcoR171 fragment derived from plasmid E43 into the filled-in Sall site of OVEC. Plasmid E43 was a kind gift of Amy S. Lee, University of Southern California, Los Angeles, CA (18). Plasmid pSP1OVEC-S containing two Sp1 binding sites derived from the HSV IE-3 promoter (19) was constructed by inserting the annealed oligonucleotides 5'-TCGAGGAGCTGTCCGAGGTGCT-3' and 5'-TCGACGTTTCAGCACCA-3' into the Sall site of pOVEC-S. REST binding motifs were generated by subcloning of the synthetic oligonucleotides 5'-TCGACGTTTCAGCACCA-3' and 5'-TCGACGTTTCAGCACCA-3' into the Sall site of pOVEC-S. REST binding motifs were generated by subcloning of the synthetic oligonucleotides 5'-TCGACGTTTCAGCACCA-3' and 5'-TCGACGTTTCAGCACCA-3' into the Sall site of pOVEC-S. Plasmid pCMV5. The myc tag was introduced by inserting the annealed oligonucleotides 5'-TCGACGTTTCAGCACCA-3' and 5'-TCGACGTTTCAGCACCA-3' into the Sall site of pOVEC-S. Plasmid pCMV5. The myc tag was introduced by inserting the annealed oligonucleotides 5'-TCGACGTTTCAGCACCA-3' and 5'-TCGACGTTTCAGCACCA-3' into the Sall site of pOVEC-S.

Expression Constructs—Cloning of the human REST cDNA was described elsewhere.2 Plasmid pCMVmycREST, encoding the full-length REST protein and in Vitro DNA-Protein Binding Assays—Expression and purification of GST-fusion proteins were done as described (24). The expression plasmid pGEX-REST was constructed by inserting a 1621 bp fragment of the REST cDNA into the filled-in XhoI site of plasmid pGEX-RG. The fusion protein encoded REST amino acids 154 to 440, encompassing the cluster of eight zinc fingers. Two additional GST expression vectors were generated encoding amino acids 953 to 1097 and 1051 to 1097. The expressed fusion proteins contained the single zinc finger motif located at the C terminus of REST. Electrophoretic mobility shift assays were performed by using 50 μg using a 7.5% gel and transferred to nitrocellulose membranes (pore size 0.2 mm). Blots incubated with anti-REST antisera were developed using an alkaline phosphatase-conjugated secondary antibody. Blots probed with antibody 9E10 were developed using the ECL system (Amersham Pharmacia Biotech).

RESULTS

Expression of REST in NS20Y and NG108-15 Cells Repressed Synapsin I Promoter Activity—We reported recently that the REST binding motif is crucial for the neuron-specific transcription of the synapsin I gene (7). To confirm that REST is the major regulator of synapsin I gene transcription, transfection experiments were performed. The expression vector pCMVmycREST was constructed encoding human REST with an N-terminal myc tag under the control of the CMV immediate-early gene promoter/enhancer (Fig. 1A). The vector was first tested in COS cells. REST migrates in SDS-polyacrylamide gel electrophoresis at ~200 kDa as shown by Western blot analysis using antibodies against REST and the myc tag (Fig. 1B). The pCMV-mycREST expression vector was subsequently used in transfection experiments together with synapsin I promoter/β-globin constructs as reporters. Plasmid pSYOVEC contained the synapsin I promoter sequence from 422 to 22, which has been shown to be necessary and sufficient for neuron-specific gene transcription (7). As a control for REST function, a deletion from −234 to −200 was introduced into the synapsin I promoter-forming plasmid pSYOVECΔNRSE. The deletion included the binding site REST had no effect upon transcription from a synapsin I promoter sequence lacking the REST binding motif is crucial for the neuron-specific transcription of the synapsin I gene (7). To confirm that REST is the major regulator of synapsin I gene transcription, transfection experiments were performed. The expression vector pCMVmycREST was constructed encoding human REST with an N-terminal myc tag under the control of the CMV immediate-early gene promoter/enhancer (Fig. 1A). The vector was first tested in COS cells. 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**Fig. 1** Expression of REST in NS20Y and NG108-15 cells abolished neuron-specific gene transcription of a synapsin I promoter/β-globin gene in NS20Y and NG108-15 cells. A, expression vector pCMVmycREST. B, Western blot analysis of COS cells transfected with pCMVmycREST. As a control, extracts from mock-transfected COS cells were analyzed. Western blots were probed with an anti-REST antiserum (left) and an antibody against the myc tag (right), respectively. C, reporter plasmids containing (pSyOVEC) or lacking (pSyOVEC\(\Delta\)NRSE) the REST binding side within the synapsin I promoter. D, reporter plasmids pSyOVEC and pSyOVEC\(\Delta\)NRSE, the ICP0ref internal standard plasmid, the empty expression vector pCMV5 (−) or the REST expression vector pCMVmycREST (+) were introduced into NS20Y (left) and NG108-15 cells (right). Transcription was analyzed by RNase protection mapping of the β-globin mRNA isolated from the transfected cells. The bands labeled test indicate correctly initiated β-globin transcripts, and the bands labeled ref were generated by the internal standard plasmid ICP0ref. RT indicates incorrectly initiated read-through transcripts of the test templates. Also shown is an aliquot of undigested cRNA (riboprobe). Size marker HaeIII-digested pBR322 is shown in lane M.

**moter with Engineered REST Binding Sites Derived from Five Neuronal Genes**—The REST binding sites termed NRSEs have been identified in neuronal genes at various locations, i.e., in promoter and enhancer positions, in the 5′-untranslated region as well as in introns. The NRSEs derived from the genes encoding synapsin I, SCG10, α₁-glycine receptor, the β2-subunit of the neuronal nicotinic acetylcholine receptor, and the m4-subunit of the muscarinic acetylcholine receptor were tested to determine whether they display similar biological activities. All sequences fit with the NRSE consensus NNCAGCACCNGCAGCNNC (14) as shown in Fig. 2A. The main difference between these NRSEs is their location within the transcription unit. Moreover, the orientation of the NRSE derived from the α₁-glycine receptor is opposite to the NRSEs found in the synapsin I, SCG10, m4 muscarinic acetylcholine receptor, and the neuronal nicotinic acetylcholine receptor β2-subunit genes. To test the biological activity of these NRSEs we decided to use model promoters to exclude influences from other neuron-specific elements. The transcription unit contained the β-globin gene as reporter. The enhancer of the glucose-regulated protein 78 (grp78) gene was inserted upstream of the TATA box. The grp78 gene is transcribed constitutively in many different tissues and cell types (27) and is not regulated by REST. Upstream of the grp78 enhancer, two copies of the different NRSEs were inserted (Fig. 2B). The reporter plasmids, the internal reference plasmid ICP0ref, and the REST expression vector pCMVmycREST (+) or the empty vector pCMV5 (−) were transfected into NS20Y and NG108-15 cells. Transcription of the β-globin reporter gene was analyzed by RNase protection mapping. The effect of REST upon transcription of the grp78 gene is shown on the left hand side (see plasmid pgrpOVEC). The presence of REST binding sites, however, together with expression of REST via transfection of a REST expression vector caused a striking decrease in transcription. This effect could be observed in NS20Y as well as in NG108-15 cells. No major differences were detected between the NRSEs tested. Thus, the NRSEs derived from the genes encoding synapsin I, SCG10, α₁-glycine receptor, the β2-subunit of the neuronal nicotinic acetylcholine receptor, and the m4-subunit of the muscarinic acetylcholine receptor are functionally identical in recruiting REST and by that means, lead to repression of grp78 enhancer-stimulated transcription.

**REST Repressed Transcription at a Distance—Putative REST binding sites have been identified in introns of various neuronal genes, most prominent in the first intron of the Ng-CAM gene where five NRSEs were found (3). An NRSE was also detected within the second intron of the L1 gene (5). To address the question if REST is able to repress transcription from distant positions, a reporter plasmid was constructed containing the β-globin reporter gene and the grp78 promoter/ enhancer. REST binding sites derived from the synapsin I gene were inserted in both orientations 2.3 kilobase pairs downstream of the transcriptional start site (Fig. 3A). The reporter plasmids, the internal reference plasmid ICP0ref, and the REST expression vector pCMVmycREST (+) or the empty vector pCMV5 (−) were introduced into NS20Y cells, and transcription was measured by RNase protection mapping of the β-globin mRNA. REST did not influence the activity of the grp78 enhancer (Fig. 3B, plasmid pgrpOVEC). The presence of REST binding sites 2.3 kilobase pairs downstream of the cap site together with synthesized REST protein led to the transcriptional repression of grp78 enhancer activity. This effect was independent of the orientation of the NRSE (Fig. 3B, plasmids pgrpOVEC\(\Delta\)syINRSE\(\Delta\)(s) and pgrpOVEC\(\Delta\)syINRSE\(\Delta\)(as)).**

Next, we tested whether REST is also able to repress an enhancer located at a distant remote position. The reporter
plasmid pSyINRSE2Sp12OVEC-S contained the β-globin gene, a TATA box, and two copies of an Sp1 binding site derived from the HSV IE-3 promoter. The SV40 enhancer was inserted downstream of the β-globin open reading frame. This enhancer in a remote position is inactive unless there is at least one copy of an upstream factor binding site in addition to the TATA box (28). Thus, the SV40 enhancer together with two Sp1 binding motifs in the promoter generated a strong transcriptional activation potential as seen in Fig. 3D (plasmid pSyINRSE2OVEC-S). To test the biological activity of REST two copies of the synapsin I or SCG10 gene, NRSEs were inserted into the reporter construct upstream of the Sp1 binding sites (Fig. 3C). Expression of REST in NS20Y cells had no impact on transcription of the reporter gene pSp12OVEC-S that lacked REST binding sites (Fig. 3D). This was confirmed by using a BAS1000 Bioimaging analyzer. Quantification of the amount of β-globin mRNA relative to the signal generated from the internal reference plasmid ICP0ref revealed that REST did not influence transcription mediated by the SV40 enhancer together with Sp1 (data not shown). This result is in agreement with in vitro DNA-protein binding assays showing that Sp1 did not interfere with the DNA binding activity of REST (Fig. 5, see below). The presence of REST binding sites in the transcription unit, however, led to a striking reduction in β-globin mRNA synthesis due to the repressor effect of REST (Fig. 3D, plasmids pSyINRSE2Sp12OVEC-S and pSCG10NRSE2Sp12OVEC-S). No functional differences were observed between the NRSEs derived from the synapsin I or SCG10 genes. We conclude that REST, in addition to repressing transcription from distant positions, is also able to block the activity of an enhancer in a remote position.

REST Did Not Display Transcriptional Activator Activity When Bound Close to a TATA Box—It was recently proposed that REST represents a dual activator/repressor molecule dependent upon the location of the REST binding site. REST was proposed to work as a transcriptional activator when the NRSE was either in the 5′-nontranslated region or less than 50 nucleotides upstream from the TATA box (13). To confirm these results, reporter plasmids were constructed that had two or four copies of the synapsin I gene NRSE in their regulatory regions (plasmids pSyINRSE2OVEC and pSyINRSE4OVEC, Fig. 4A). The spacer between the NRSE and the TATA box was 12 nucleotides. The reporter plasmids, the ICP0ref internal reference plasmid, and the REST expression vector were transfected into NS20Y cells, and transcription was measured by RNase protection assay. No transcriptional activation could be observed when REST was synthesized in NS20Y cells via transfection of a REST expression vector (Fig. 4B). As a control, plasmid pSyINRSE2Sp12OVEC-S was included in the assay showing the repressor effect of REST upon a transcription unit composed of the SV40 enhancer and two Sp1 binding sites. In support of this, no activation of transcription was measured in CHO-K1 cells using the reporter plasmids pSyINRSE2OVEC and pSyINRSE4OVEC (data not shown). In CHO-K1 cells, an endogenous REST-like activity is present as shown in the analysis of the synapsin I promoter (7). Thus, the endogenous REST activity in CHO-K1 as well as REST activity generated by transfection in neuroblastoma cells did not promote transcription of the reporter genes containing REST binding sites in proximity to the TATA box. From these data we conclude that

C, reporter plasmids, the ICP0ref internal standard plasmid, and either the empty expression vector pCMV5 (-) or the REST expression vector pCMVMycREST (+) were introduced into NS20Y and NG108-15 cells. Transcription was analyzed by RNase protection mapping of the β-globin mRNA isolated from the transfected cells. The bands are labeled as in Fig. 1. For definitions of test and ref, see legend to Fig. 1.
REST by itself has no transcriptional activator properties.

The N-terminal Zinc Finger Cluster of REST Functioned as the DNA Binding Domain for Neuronal Genes—The N-terminal zinc finger domain of REST was expressed in Escherichia coli as a fusion protein with S. japonicum GST, and the recombinant protein was used for in vitro DNA-protein binding experiments (Fig. 5). These experiments showed that REST binds to the NRSE of the human synapsin I promoter as well as to the NRSE derived from the SCG10 gene. This interaction could be competed by adding an excess of cold probe and also by adding DNA containing a homologous motif derived from the SCG10 or synapsin I promoter, respectively. No competition was observed with DNA binding sites for the zinc finger transcription factors Sp1 and egr-1, indicating the specificity of the DNA binding activity of REST. No competition was obtained with an unrelated transcription factor binding site, the cAMP response element. In contrast, a fusion protein encoding GST together with the C-terminal zinc finger motif of REST did not bind NRSEs derived from the synapsin I or SCG10 gene (data not shown). Furthermore, no DNA-protein interaction could be detected between the N-terminal zinc finger cluster or the C-terminal zinc finger motif of REST with an X2-box DNA probe derived from the major histocompatibility complex class II DPA gene promoter as proposed (25). Rather, the C-terminal zinc finger motif exhibited transcriptional repressor activity instead of DNA binding activity as shown below.

REST Contains Two Transferable Repression Domains That Functioned from Sites Near and Far—To locate the REST...
repressor domain(s), various regions of REST were expressed as fusion proteins with the DNA binding domain of the yeast transcription factor GAL4 (Fig. 6). The reporter plasmids pSyINRSE<sup>2</sup>OVEC and pSyINRSE<sup>4</sup>OVEC containing the rabbit β-globin reporter gene, a TATA box, and two or four binding sites for REST derived from the synapsin I promoter upstream of the TATA box. Plasmid pSyINRSE<sup>2</sup>Sp<sup>1</sup>OVEC-S was included in the assay as a control. B, reporter plasmids, the ICPOref internal standard plasmid, and either the empty expression vector pCMV5 (--) or the REST expression vector pCMVmycREST (+) were introduced into NS20Y. Transcription was analyzed by RNase protection mapping of the β-globin mRNA isolated from the transfected cells. For definitions of RT, test, and ref, see legend to Fig. 1. M, size marker HaeIII-digested pBR322 is shown in lane M.

**Fig. 4.** REST displayed transcriptional repression but not transcriptional activation activity. A, reporter plasmids pSyINRSE<sup>2</sup>OVEC and pSyINRSE<sup>4</sup>OVEC containing the rabbit β-globin reporter gene, a TATA box, and two or four binding sites for REST derived from the synapsin I promoter upstream of the TATA box. Plasmid pSyINRSE<sup>2</sup>Sp<sup>1</sup>OVEC-S was included in the assay as a control. B, reporter plasmids, the ICPOref internal standard plasmid, and either the empty expression vector pCMV5 (--) or the REST expression vector pCMVmycREST (+) were introduced into NS20Y. Transcription was analyzed by RNase protection mapping of the β-globin mRNA isolated from the transfected cells. For definitions of RT, test, and ref, see legend to Fig. 1. M, size marker HaeIII-digested pBR322 is shown in lane M.

reporter plasmids pUAS<sup>5</sup>grpOVEC and pUAS<sup>5</sup>SV40OVEC (Fig. 7, B and D) encompassing the N-terminal 152 and the C-terminal 144 amino acids of REST. Thus, the REST molecule contains two distinct repressor domains that were transferable to a heterologous DNA binding domain and functioned upon strong viral or eukaryotic promoters. A weak transcriptional repressor activity was generated by GST-REST fusion proteins 4 to 6, all containing the repeat region of REST. Expression of the GAL4-REST fusion protein 8, however, which also contained this region, had no effect upon transcription, arguing against a general role of the repeat region in transcriptional repression. Moreover, the repressor activity of GST-REST fusion proteins 4 to 6 was negligible in comparison to the GST-REST fusion proteins 1 and 9 containing the N- and C termini of REST.

To test whether these major repression domains were functional not only from proximal but also from distal positions, two additional reporter plasmids were constructed (Fig. 8 A). Plasmid pUAS<sup>5</sup>Sp<sup>1</sup>OVEC-S contained the SV40 enhancer and two Sp1 binding sites as described for plasmids pSyINRSE<sup>2</sup>Sp<sup>1</sup>OVEC-S and pSCG10NRSE<sup>2</sup>Sp<sup>1</sup>OVEC-S (Fig. 3 C). The NRSE motifs were changed to UAS to allow binding of the GAL4-REST fusion proteins. The reporter plasmid pUAS<sup>5</sup>1105-SV40OVEC contained the rabbit β-globin reporter gene, a TATA box, the SV40 promoter, and five binding sites for GAL4. The UAS motifs and the SV40 promoter were separated by 1105 nucleotides. The reporter plasmids and the expression plasmids pGAL4-R1 and pGAL4-R9 encoding the N- and C-terminal repressor domains of REST together with the GAL4 DNA binding domain and the ICPOref internal reference plasmid were transfected into NS20Y cells, and transcription was measured by RNase protection assay (Fig. 8 B). Both GAL4-REST fusion proteins repressed transcription mediated by Sp1 together with SV40 enhancer-binding proteins (reporter plasmid pUAS<sup>5</sup>Sp<sup>1</sup>OVEC-S), indicating that both repressor domains of REST were able to repress transcription of a strong...
enhancer in remote position (Fig. 8B, left panel). Both repressor domains also blocked transcription of the SV40 promoter that was located 1105 nucleotides downstream of the binding site of the GAL4-REST fusion proteins (Fig. 8B, right panel). The N-terminal repression domain of REST showed a slightly higher activity in repressing transcription of the SV40 promoter than the C-terminal repression domain, suggesting that both repressor domains of REST are distinct and differ not only in their primary structure but also in their biological activity.

**DISCUSSION**

The REST binding motif within the synapsin I promoter is crucial for neuron-specific gene transcription. Deletion of the REST binding site abolished neuron-specific expression of the reporter gene entirely, allowing constitutively acting elements of the promoter to direct expression in a nontissue-specific manner (7). These experiments revealed that the synapsin I promoter worked perfectly in nonneuronal cells. However, the NRSE had to be deleted to allow transcription, indicating that the presence of REST in those cells normally represses synapsin I promoter activity. The negative regulatory mechanism of synapsin I transcription was further supported by experiments reported here, showing that expression of REST in neuroblastoma cells by transfection of a REST expression vector blocked transcription of the reporter gene under control of synapsin I promoter sequences. REST had no effect upon transcription from a synapsin I promoter/reporter gene when the REST binding site had been deleted. We have shown recently that the expression pattern of synapsin I and REST in human neuroblastoma cells was inverse. REST and synapsin I were, however, coexpressed in the neuroblastoma cell lines tested, although at different concentrations, indicating that low levels of REST are tolerable for allowing neuron-specific gene transcription. Thus, the concentration of REST is important for REST...
REST is a transcriptional repressor that is able to block transcription mediated by strong heterologous promoters of viral and eukaryotic origin such as the grp78 promoter/enhancer or the SV40 promoter. REST repressed transcription independent of the nature of the heterologous promoter. Moreover, we showed that REST also repressed transcription despite the location or orientation of its binding site within a gene. REST blocked transcription of a viral promoter from a remote position, downstream of the open reading frame. Similarly, the activity of a strong enhancer in remote position was repressed by REST bound several kilobases upstream of the enhancer in the promoter region. Thus, REST fulfills the criteria of a transcriptional silencer-binding protein that blocks transcription irrespective of the location or orientation to the enhancer and the promoter. The positions of naturally occurring REST binding sites at different sites within neuron-specific genes is consistent with the ability of these motifs to silence transcription from any position within a transcription unit. As already mentioned, NRSEs were identified not only in promoter and enhancer positions but also in the 5′-untranslated region as well as in introns, suggesting that the actual binding position within a neuronal gene plays obviously no role for the biological activity of REST. In this study, five NRSEs were analyzed derived from genes encoding synapsin I, SCG10, α1-glycine receptor, the β2-subunit of the neuronal nicotinic acetylcholine receptor, and the m4-subunit of the muscarinic acetylcholine receptor. All of these motifs fit with the proposed consensus sequence NNCAAGCCNNGACAGNN (14).

No differences in the biological activities of these NRSEs were detected, although in their natural context, they differ in their orientation and location. The NRSE of the α1-glycine receptor gene affected a heterologous promoter in a manner indistinguishable from the other NRSEs, although in the α1-glycine receptor gene it is found in the 5′-untranslated region in inverse orientation to the synapsin I gene NRSE. We conclude that a NRSE functions as a REST binding motif independent of orientation and location as long as it fits with the proposed core NRSE.

Recently it had been proposed that REST is a dual enhancer/silencer-binding protein dependent on the location of the REST binding site. In particular, transcriptional activation of REST should take place in promoters with a REST binding site close to the TATA box (13). Three lines of evidence argue against a role of REST in transcriptional activation. First, transcription of a reporter gene containing NRSEs in proximity to the TATA box was not activated by REST overexpressed in the neuroblastoma cell line NS20Y. Second, the same transcription unit was inactive in CHO-K1 cells exhibiting an endogenous REST-like gene. Moreover, those proteins are ubiquitously expressed, indicating that the promoter context of their target genes determines the specificity of activating or repressing transcription. In contrast, the expression levels of REST vary between different cell types, and the actual REST concentration determines whether REST is able to repress transcription of its target genes. Thus, the specificity of REST function is determined by the REST concentration and not by the promoter context. As shown and discussed here, the location of the REST binding site within a transcription unit is not important for the biological activity of REST.

A dissection of important domains of REST identified the DNA-tethering domain that anchors the protein to DNA and two repressor domains located at the N and C termini of the molecule. No activation domain could be detected. The repressor domains located at the N and C termini were transferable to the heterologous DNA binding domain of GAL4 and functioned when bound to distal and proximal positions within a gene. We were unable to detect any DNA binding activity of the zinc finger motif located at the C terminus of REST using either NRSEs or the X2-box of the major histocompatibility complex class II DPA gene, suggesting that this domain is a transcriptional repressor domain and not a DNA binding domain (25). Two repressor domains located on both termini of the REST molecule were also identified by others (33). REST is thus comparable with the thyroid hormone receptor or the zinc...
finger proteins containing Krüppel-associated boxes (34, 35). All of these proteins contain powerful repressor domains that work perfectly with a heterologous DNA binding domain. REST is unique in having two repressor domains that are clearly distinct in their primary structure. In addition, we found that the C-terminal repressor domain is not as active as the N-terminal repressor domain in repressing transcription from a distant position. In support of this, we recently found that both repressor domains of REST have to interact with distinct nuclear factors to repress transcription, indicating that two different modes of action were used by REST to repress transcription. How the transcriptional repressor REST works on the molecular level is currently under investigation in the laboratory.

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