The synapsins are a family of neuronal phosphoproteins that selectively bind to small synaptic vesicles in the presynaptic nerve terminal. The human synapsin I gene was functionally analyzed to identify control elements directing the neuron-specific expression of synapsin I. By directly measuring the mRNA transcripts of a reporter gene, we demonstrate that the proximal region of the synapsin I promoter is sufficient for directing neuron-specific gene expression. This proximal region is highly conserved between mouse and human. Deletion of a putative binding site for the zinc finger protein, neuron-restrictive silencer factor/RE-1 silencing transcription factor (NRSF/REST), abolished neuron-specific expression of the reporter gene almost entirely, allowing constitutively acting elements of the promoter to direct expression in a non-tissue-specific manner. These constitutive transcriptional elements are present as a bipartite enhancer, consisting of the region upstream (nucleotides −422 to −235) and downstream (nucleotides −199 to −143) of the putative NRSF/REST-binding site. The latter contains a motif identical to the CAMP response element. Both regions are not active or are only weakly active in promoting transcription on their own and show no tissue-specific preference. From these data we conclude that neuron-specific expression of synapsin I is accomplished by a negative regulatory mechanism via the NRSF/REST binding motif.

The synapsins are a family of neuronal phosphoproteins that coat the cytoplasmic surface of small synaptic vesicles (Thiel, 1993). This family consists of four proteins, synapsin Ia and synapsin Ib (collectively termed synapsin I) and synapsin IIa and synapsin IIb (collectively termed synapsin II). Synapsins I and II are generated via alternative splicing from two different genes (Südhof et al., 1989). Molecular cloning of bovine, human, and rat synapsins revealed striking homologies in the amino-terminal 420 amino acids of all four synapsins. The major difference between synapsins I and II lies in the C-terminal domain of the synapsin I isoforms. This domain contains clusters of basic amino acids as well as two recognition sites for Ca²⁺/calmodulin-dependent protein kinase II (Südhof et al., 1989).

Synapsin I has been postulated to link synaptic vesicles to the cytoskeleton, thus regulating the availability of synaptic vesicles for exocytosis (Greengard et al., 1993). In addition, a role for synapsin I in the regulation of short term plasticity has been suggested (Rosahl et al., 1993). Mice lacking synapsin I or both synapsins I and II are viable and fertile with no gross anatomical abnormalities. These mice, however, frequently experience seizures, indicating the essential functions of the synapsins in synaptic vesicle regulation (Rosahl et al., 1995).

Virtually all neurons express the synapsins (Südhof et al., 1989) and there are no non-neuronal counterparts known for the synapsins, in contrast to the synaptic vesicle proteins synaptobrevin, synaptophysin, and synaptotagmin (Zhong et al., 1992; McMahon et al., 1993; Li et al., 1995). The restricted expression of synapsins I and II in the nervous system establishes the synapsin genes as good candidates for an investigation of neuron-specific gene expression. The rat and human synapsin I genes have been analyzed, and it was shown that the 5′-flanking region is sufficient for neuron-specific expression (Sauerwald et al., 1990; Thiel et al., 1991). The synapsin I gene promoter contains a sequence motif similar to the neuron-restrictive silencer element/repressor element-1 (NRSE/RE-1) of the SCG10 and type II sodium channel gene, respectively (Kraner et al., 1992; Mori et al., 1992). This element was suggested to function as a binding site for a protein that is expressed only in non-neuronal cells. This NRSE/RE-1-binding protein was proposed to shut down the activity of a constitutive enhancer upon binding to the NRSE/RE-1 sequence. A zinc finger protein termed neuron-restrictive silencer factor (NRSF)/RE-1 silencing transcription factor (REST) was recently discovered that binds to this motif and functions as a transcriptional repressor (Chong et al., 1995; Schoenherr and Anderson, 1995). A homologous motif present in the synapsin I promoter was shown to function in silencing synapsin I gene expression in non-neuronal cells (Li et al., 1993). However, because deletion or mutation of this motif still showed preferential expression of synapsin I promoter-reporter genes in neuronal cells relative to non-neuronal cells, it was suggested that an additional cis-acting element in the synapsin I promoter is necessary for neuron-specific gene expression (Li et al., 1993). To further investigate the mechanisms involved in the neuron-specific expression of synapsin I, the proximal part of the 5′-flanking region was functionally analyzed. Here, we present data showing that the negative regulatory mechanism via the NRSE/RE-1 sequence is solely responsible for restricting the expression of synapsin I to neuronal cells.

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1The abbreviations used are: NRSE, neuron-restrictive silencer element; CRE, cyclic AMP response element; NRSF, neuron-restrictive silencer factor; RE-1, repressor element 1; REST, RE-1 silencing transcription factor; RSV, Rous sarcoma virus; GABA, γ-aminobutyric acid A; CHO, Chinese hamster ovary.
**EXPERIMENTAL PROCEDURES**

**DNA Cloning**—A genomic clone termed pSyC-1–9a (Rosahl et al., 1993) containing the mouse synapsin I promoter region was kindly provided for us by Thomas Südhof, University of Texas, Dallas.

**Reporter Constructs**—Plasmids OVEC and ICPOref have been described (Westin et al., 1987; Thiel et al., 1994). Plasmids A, B, C, D, E, F, and G containing synapsin I promoter-β-globin reporter genes were generated by inserting the following synapsin I promoter fragments into the Sal I or Sal I–Ecl ISI sites of OVEC: plasmid A, 22356 to 222; plasmid B, 2422 to 222; plasmid C, 2422 to 235; plasmid D, 2234 to 22; plasmid E, 199 to 22; plasmid F, 142 to 22; and plasmid G, 115 to 22. The plasmid B contains a deletion of synapsin I promoter sequences from 2234 to 2200, generated with the restriction enzymes AluI and BstNI. The plasmid pRSVOVEC-1 was constructed by inserting a Bsu36I–EcoRI fragment of the RSV long terminal repeat (sequence from 273 to 49) into the filled in SalI site of OVEC.

**Electrophoretic Mobility Shift Assay**—Binding assays were performed as described (Thiel et al., 1994). To generate the probes, the following digononucleotides were annealed: human synapsin I promoter (–283 to –267), 5′-TCGAGAGGAGGGGGAGAAG-3′ and 5′-TCGACTTTCCCCCTCCTTCTC-3′; consensus sequence of six purine-rich motifs present in the rat GABAA receptor d subunit gene promoter, 5′-TCGAGAGGAGGGGGAG-3′ and 5′-TCGACTCTCCTCCTCCCCC-3′ (Motejlek et al., 1994); rat Na,K-ATPase a3 subunit gene promoter (–143 to –124), 5′-TCGAGGTGAAGGGGGAAG-3′ and 5′-TCGACTCCCCCTTCCCCCTTCA-3′ (Pathak et al., 1994); mouse neurofilament heavy gene promoter (–107 to –70), 5′-TCGAGAGGAGGGGAAGGCTGAG-3′ and 5′-TCGACTGAGGGAGGCCCCACCCTCTTCTTCCCTTCCC-3′ (Schwartz et al., 1994); mouse secretogranin II promoter (–74 to –52), 5′-TCGAGAGGAGGGGAGGCTGAG-3′ and 5′-TCGACTTCCCCCTCCTGAGGCCCCGCTTCTC-3′ (Schimmel et al., 1992). All probes contained XhoI and SalI restriction sites for radiolabeling.

**Miscellaneous Techniques**—Chinese hamster ovary cells (CHO-K1,
Comparison of the 5' flanking Region of the Human and Mouse Synapsin I Gene—The sequences of the 5' flanking regions of the human and mouse synapsin I genes are shown in the upper panel of Fig. 1. The proximal region of the synapsin I promoter sequence is highly conserved between mouse and human including the transcriptional initiation site and the consensus binding sites for the transcription factors CREB (CRE), zif268/egr-1, and NRSF/REST. A dot matrix analysis between the synapsin I promoter of human and mouse, depicted in the lower panel of Fig. 1, revealed that the homology is restricted mainly to the proximal 400 base pairs upstream of the transcriptional start site. We conclude that the proximal region of the synapsin I promoter contains functionally important regulatory motifs due to the high evolutionary conservation of these sequences.

The Proximal Region of the Synapsin I Promoter Directs Neuron-specific Gene Transcription—To confirm that the proximal region of the synapsin I promoter contains neuron-specific and/or constitutive transcriptional elements, a fragment of the human synapsin I promoter from –422 to –22 was inserted into a β-globin expression vector (plasmid B). Fragments encompassing synapsin I promoter sequences from –2352 to –22 and from –115 to –22 were cloned into the same vector (plasmids A and G) and served as positive and negative controls, respectively, for neuron-specific gene transcription, as determined from previous studies (Thiel et al., 1991). Plasmid pRSVOVEC-1 containing the β-globin gene under control of the RSV long terminal repeat was used to measure the activity of a strong tissue-unspecific promoter. These plasmids, depicted in Fig. 2A, were transiently transfected into the neuronal cell line NG108-15 and the non-neuronal cell line CHO-K1 together with plasmid ICP0ref, a mutated β-globin gene under the control of the herpes simplex virus ICP0 gene promoter, to correct for variations in transfection efficiencies. 48 h post-transfection, reporter plasmids were introduced into NG108-15 and CHO-K1 cells. Cytoplasmic RNA was measured by RNA protection assay. 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 markers, BstEII cut DNA (left) and HaeIII-digested pBR322, are shown in lane M.

The sequence homologous to the NRSE/RE-1 is necessary and sufficient for directing neuron-specific expression of synapsin I. A, sequence of the NRSE/RE-1 derived from the SCG10 and sodium channel genes and homologous sequences from the genes encoding synapsin I and the Na,K-ATPase α3 subunit, respectively. B, reporter plasmids containing (plasmid B) or lacking (plasmid BΔ) the NRSE/RE-1 homologous sequence of the synapsin I promoter. C, RNA protection mapping of β-globin mRNA isolated from transfected NG108-15, NS20Y, HepG2, CHO-K1, and HeLa cells. The data are presented in the same manner as in Fig. 2.
transfection, cytoplasmic RNA of the transfected cells was isolated, hybridized to a β-globin derived cRNA probe, and analyzed by RNase protection mapping (Fig. 2B). The synapsin I promoter regions present in plasmids A and B directed expression of the reporter gene only in NG108–15 cells, indicating that the proximal region of the synapsin I promoter contains neuron-specific elements. The synapsin I promoter region from −115 to −22 showed no transcriptional activity in either cell line, suggesting that this region does not on its own play a role in the regulation of transcription of the synapsin I gene. The RSV enhancer was active in both cell types. These data show that the proximal region of the 5′-flanking region of the human synapsin I gene is sufficient for directing neuron-specific gene expression.

Deletion of the Putative Binding Site for NRSF/REST Abolishes Neuron-specific Gene Expression of a Reporter Gene—It has been reported that a sequence motif homologous to the NRSE/RE-1 sequence present in the SCG10 and type II sodium channel gene (Fig. 3A) plays a role in regulating neuron-specific expression of synapsin I. However, deletion or mutation of the NRSE/RE-1 homologous sequence still allowed preferential expression of the synapsin I promoter-reporter genes in neuronal cells relative to non-neuronal cells. It was therefore suggested that the NRSE/RE-1 sequence was not solely responsible for the neuron-specific expression of synapsin I and that an additional cis-acting sequence was necessary for this regulation (Li et al., 1993). To test whether there are other cis-acting elements necessary and to confirm that the NRSE/RE-1 homologous sequence alone did not confer full neuron-specific expression, a deletion from −234 to −200 was introduced into the synapsin I promoter sequence in plasmid B, thus generating plasmid BΔ (Fig. 3B). This plasmid and plasmid B were introduced into the neuronal cell lines NG108–15 and NS20Y as well as in the non-neuronal cell lines HepG2, CHO-K1, and HeLa. NG108–15 and NS20Y cells had previously been shown to express endogenous synapsin I (Petersohn et al., 1995). Fig. 3C shows that the region from −22 to −22 of the synapsin I promoter directs transcription of the β-globin gene in NS20Y and NG108–15 cells, whereas no transcripts could be detected in CHO-K1, HepG2, and HeLa cells (plasmid B). A deletion of the putative NRSF/REST-binding site had no effect upon transcription in the neuronal cells (plasmid BΔ) and virtually abolished neuron-specific expression of the reporter gene, allowing β-globin transcripts to be detected in the non-neuronal cell lines CHO-K1, HepG2, and HeLa. A quantitative estimation of the level of transcription by PhosphorImager analysis normalized to the reference transcripts revealed that as a result of deleting the NRSE/RE-1 sequence, transcription in HepG2 and HeLa cells was comparable with that in NG108–15 and NS20Y cells. Transcription of the β-globin reporter gene in CHO-K1 cells was somewhat lower, possibly reflecting a species difference.

The Synapsin I Promoter Region from −199 to −143 Functions as a Weak Constitutive Enhancer Element—The fact that the NRSE/RE-1 sequence is crucial for neuron-specific gene expression suggests two questions. First, is the NRSE/RE-1 the only sequence motif directing neuron-specific gene expression of synapsin I? If this is the case, a negative regulatory mechanism would be decisive for the neuron-specific expression of synapsin I. Second, the NRSE/RE-1-binding protein is suggested to shut down a constitutive enhancer in non-neuronal cells, thus constitutive enhancer elements have to be localized in the proximal region of the synapsin I promoter. To answer both questions, a deletion analysis of the synapsin I promoter...
region from −199 to −22 was performed (Fig. 4A, plasmids B, D, E, F, and G). At least one cis-acting element necessary for neuron-specific gene transcription was proposed in this region (Li et al., 1993). Deletion mutants were introduced into NS20Y, NG108–15, CHO-K1, and HepG2 cells, and transcription was monitored by mapping of the β-globin transcripts (Fig. 4B). The synapsin I promoter region from −142 to −22 (plasmid F) could not activate transcription of the reporter gene in both neuronal and non-neuronal cells, indicating that this region does not, at least, on its own, activate transcription. In contrast, plasmid E that contained the promoter region from −199 to −22 was active in all tested cell types, suggesting that constitutive cis-acting elements are located between −199 and −142 of the synapsin I promoter. Transcriptional activation of the reporter gene, directed by synapsin I promoter sequences from −199 to −22, was much lower than that measured for synapsin I promoter sequences from −422 to −22 (compare the signals generated by plasmids B and E). This indicates that the region from −199 to −143 is not alone responsible for constitutive transcription of the synapsin I gene.

Plasmids B and D contain the synapsin I promoter sequences from −422 to −22 and −234 to −22, respectively, including the motif homologous to the NRSE/RE-1 sequence. As expected, transcription of the β-globin gene was restricted to the neuronal cell lines NS20Y and NG108–15. However, transcriptional induction directed from the longer promoter fragment in plasmid B was much stronger than that from the shorter one in plasmid D, indicating that the promoter region from −422 to −235 contributes to the transcription of the synapsin I gene. From these data, we conclude that neuron-specific gene expression is mediated solely through negative regulation by the homologous NRSE/RE-1 sequence and that constitutive cis-acting elements are present within a bipartite enhancer structure consisting of synapsin I promoter region from nucleotides −199 to −143 and from nucleotides −422 to −235.

The Synapsin I Promoter Region from −422 to −235 Does Not Function as Either Tissue-specific or Constitutive Enhancer Element on Its Own—To test whether the synapsin I promoter region from −422 to −235 contains cis-acting elements for stimulating transcription, plasmids were introduced into NS20Y, NG108–15, CHO-K1, and HepG2 cells that contained synapsin I promoter sequences from −422 to −22 and −422 to −235, respectively (plasmids B and C, Fig. 5A). Surprisingly, the synapsin I promoter region from −422 to −235 did not show any effect upon transcription on its own, as depicted in Fig. 5B. We postulate that this sequence requires synapsin I promoter sequences downstream from the NRSE/RE-1 site to exhibit a stimulatory effect upon transcription.

Nuclear Proteins Bind to the Synapsin I Promoter Region from −422 to −235—The synapsin I promoter region from −422 to −235 was analyzed by DNA-protein binding assays in order to identify binding sites of sequence-specific transcription factors. This analysis discovered a purine-rich sequence motif within this region (−283 to −267) that is also present in the promoter regions of the genes encoding the GABA_α receptor δ subunit, the Na,K-ATPase α3 subunit, and the heavy neurofilament (Fig. 6A). In addition, this region was implicated as a possible cis-acting element for neuron-specific gene expression (Motejek et al., 1994; Pathak et al., 1994; Schwartz et al., 1994). Gel mobility shift assays were performed with double-stranded, synthetic oligonucleotides corresponding to the synapsin I promoter region from −283 to −267 and nuclear extracts prepared from NG108–15, NS20Y, and NIH3T3 cells. Three major DNA-protein complexes (marked with arrowheads 1, 2, and 3 in Fig. 6B) were detected with nuclear extracts prepared from the neuronal cells as well as from the non-neuronal NIH3T3 cell line (Fig. 6B, lanes 2, 5, and 8), suggesting that the proteins responsible for forming these complexes are not tissue-specific. The DNA-protein complexes were competed efficiently by addition of a 100-fold excess of unlabeled probe (Fig. 6B, lanes 3, 6, and 9). In contrast, an unrelated DNA probe failed to compete with these complexes (Fig. 6B, lanes 4, 7, and 10). To investigate whether similar proteins bind to the synapsin I promoter and the promoters of the GABA_α receptor δ subunit, the Na,K-ATPase α3 subunit, and the heavy neurofilament gene, synthetic double-stranded oligonucleotides were annealed and radiolabeled. Fig. 6C depicts the DNA-protein complexes formed with each of these probes using nuclear extracts from brain. This experiment shows that the protein(s) responsible for forming the DNA-protein complex 3 binds(s) to all four DNA probes, although with different affinities.

**DISCUSSION**

Gene expression in neurons was analyzed using the synapsin I gene as a model. The 5′-flanking region of the human synapsin I gene was studied for cis-acting elements regulating transcription by transient transfection experiments. The results presented here show that the proximal region of the synapsin I promoter, i.e. the sequence from −422 to −22, is necessary and sufficient for neuron-specific gene expression. The importance of this region is supported by a sequence comparison between the human and mouse promoter, indicating that this region has been highly conserved in evolution.

Deletion mutagenesis of the proximal synapsin I promoter revealed that a sequence element homologous to the NRSE/RE-1 of the SCG10 and type II sodium channel genes is critical for the neuron-specific expression of synapsin I. Strikingly, a
deletion of this element resulted in transcription of a reporter gene in non-neuronal cells at a level similar to that in neuronal cells. A previous report noted that this element represses synapsin I transcription in non-neuronal cells (Li et al., 1993). However, although this element was deleted or mutated, the synapsin I promoter-reporter genes were still preferentially expressed in neuronal cells, leading to the suggestion that the NRSE/RE-1 on its own was not fully responsible for the neuron-specific expression of synapsin I and that an additional element was necessary (Li et al., 1993). These data can be explained by the use of PC12 cells as the sole neuronal model system in these experiments. We have found that in contrast with other neuronal cell lines tested, PC12 cells give an exceptionally high level of transcription following transfection of promoter-reporter gene constructs, thus leading to an overestimation of transcription in these cells (Petersohn et al., 1995). Our conclusion that the NRSE/RE-1 is in fact the sole cis-acting element for directing neuron-specific expression of synapsin I is based upon the observations that 1) a deletion of this motif in a synapsin I promoter-reporter gene abolished tissue-specific transcription of the reporter and 2) no other sequence motif of the proximal synapsin I promoter region could confer neuron-specific expression to a reporter gene.

The involvement of the NRSE/RE-1 in regulating neuron-specific gene expression has been demonstrated for the genes encoding SCG10, type II sodium channel, and Na,K-ATPase α3 subunit (Kraner et al., 1992; Mori et al., 1992; Pathak et al., 1994). The gene products are members of multigene families where some of these members are expressed in non-neuronal cells. Thus, it has been postulated that a negative regulatory mechanism via the NRSE/RE-1 fits very well for the regulation of those gene families where constitutive enhancer elements are shared between single family members and the unique, neuron-specific expression of a particular gene is accomplished by repression in non-neuronal cells (Hoyle et al., 1994). Our results obtained in the analysis of synapsin I gene expression indicate that the group of genes regulated by a negative regulatory mechanism via the NRSE/RE-1 sequence has to be extended to bona fide neuronal genes. In addition, consensus NRSEs have been discovered in the genes encoding subunits of the glycine, acetylcholine, and N-methyl-D-aspartate receptors as well as in the neurofilament and neuron-specific tubulin genes, suggesting a role for the NRSE/RE-1 sequence as master negative regulatory element of neurogenesis (Schoenherr and

![Fig. 6. A common protein binds to a purine-rich sequence in the regulatory region of the genes encoding synapsin I, GABA<sub>A</sub> subunit, Na,K-ATPase α3 subunit, and the heavy neurofilament.](image)

The purine-rich motif present in the upstream regions of the synapsin I (−283 to −267), GABA<sub>A</sub> subunit (Motejek et al., 1994), Na,K-ATPase α3 subunit (−143 to −124, Pathak et al., 1994), and heavy neurofilament gene (−107 to −70, Schwartz et al., 1994) was used in the electrophoretic mobility shift assay using nuclear extracts derived from NS20Y, NG108-15, and NIH3T3 cells. The radiolabeled probe was derived from the human synapsin I promoter (sequence −283 to −267). A binding experiment without extract is depicted in lane 1. competitor DNA was added to the reaction at 100-fold (lanes 3, 4, 7, 9, and 10) molar excess to the probe. Synthetic oligonucleotides containing the CRE of the secretogranin II promoter (denoted “CRE”) were used as an unrelated competitor (lanes 4, 7, and 10). The arrowheads indicate the DNA-protein complexes 1, 2, and 3 consisting of nuclear proteins bound to the purine-rich motif of the synapsin I promoter. C, gel retardation assay with nuclear proteins of mouse brain and radiolabeled DNA probes originating from the synapsin I, GABA<sub>A</sub> subunit, Na,K-ATPase α3 subunit, and heavy neurofilament gene promoters. All probes generated a DNA-protein complex with a similar mobility than complex 3 in B.
Regulation of Synapsin I Gene Expression

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The NRSF/REST protein represses the activity of constitutive enhancers (Kraner et al., 1992; Mori et al., 1992). One cis-acting element mediating transcriptional induction in neuronal and non-neuronal cells was mapped between –199 and –142 of the synapsin I promoter consisting most likely of the CRE, that was proposed to function in the synapsin I gene as a basal transcriptional element and not as a CAMP-inducible enhancer (Jungling et al., 1994). In addition, the synapsin I promoter region from –422 to –235 was shown to participate in regulating synapsin I gene expression. However, this region could not serve as a cis-acting element on its own in transfection experiments but rather required the presence of downstream element, most likely the CRE, for its function. This is in agreement with other studies showing that enhancers and upstream promoter elements are typically composed of various sequence modules that provide binding sites for transcription factors. Binding of those factors results in a synergistic activation of transcription, i.e. the intact enhancer displays a distinct activity in comparison with the individual elements (reviewed in Müller et al., 1988; Tjian and Maniatis, 1994). In conclusion, we find that the homologous NRSF/RE-1 sequence is the sole regulatory element necessary and sufficient for neuron-specific expression of the synapsin I gene.