

## Position-dependent effect of a neural-restrictive silencer-like element present in the promoter downstream of the SCG10-like protein gene

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**Neural-restrictive silencer (NRS) has been well characterized in SCG10 and many other neuron-specific genes; it is, however, unknown whether the promoters of the SCLIP and RB3 genes (two other SCG10 family members) share basal transcriptional mechanisms with SCG10 or not. To explore how NRS-mediated neural-specific gene transcription has evolved, we determined the genomic and promoter structures of the SCLIP gene, and found that the gene retained an NRS-like element that functioned as a negative regulator in non-neuronal cells. However, unlike the NRS in the SCG10 gene, this NRS<sup>SCLIP</sup> was located downstream of the transcription start site, and showed a position-dependent repressing activity. Further gel-shift and NRS factor (NRSF) co-transfection experiments revealed that NRS<sup>SCLIP</sup> was bound and regulated by NRSF. Such an element was not found in the gene promoter of RB3, suggesting that the NRS–NRSF regulatory system evolved once SCLIP had diverged from RB3 or stathmin.**

**Keywords:** gene expression/gene regulation/neuron/repressor/silencer/transcription.

**Abbreviations:** BDNF, brain-derived neurotrophic factor; HDAC, histone deacetylase; ORF, open reading frame; PCR, polymerase chain reaction; NRD, nucleosome remodelling and deacetylation; NRS, neural-restrictive silencer; NRSF, neural-restrictive silencing factor; REST, repressor element 1-silencing transcription factor; SCG10, superior cervical ganglia clone No. 10; SCLIP, SCG10-like protein.

Transcriptional mechanisms for cell type-specific gene expression generally involve both positive and negative regulatory factors, which recruit either co-activator or co-repressor that binds to histone modifying proteins such as acetylases or deacetylase, thereby leading to chromatin remodelling (1). The chromatin structural modification triggers and stabilizes cellular differentiation status; thus the enhancer or silencer-mediated chromatin remodelling is a central mechanism of cellular specification during animal development (2).

During the mammalian nervous system development a cell-type selective negative regulator named NRSF (neural-restrictive silencing factor) (3) or REST (repressor element-1 silencing transcription factor) (4) plays pivotal role in determining neuron-specific gene expression (5–8). The transcriptional regulation by NRSF/REST is a key issue during neuronal differentiation, maturation, plasticity, diseases, as well as stem cell maintenance (9–15). The target sequence of NRSF/REST is a 22-bp-long neural restrictive silencer element (NRSE) (also called RE-1), a versatile negative-acting *cis*-regulatory element that prevents the expression of neuron-specific genes in non-neuronal cells (16–18). While the genome data survey studies of NRSE demonstrate that the total numbers of potential target genes of the NRSF/REST in the human genome exceed 1,000 genes (19–21), its exact biochemical analyses of the cell type-specific promoter repression by this factor has been elucidated for a limited set of genes including neuronal growth-associated proteins, ion channels, neuropeptides, neurotransmitter synthetic enzymes, neurotransmitter receptors, neurotrophins and their receptors (22–37, also see reviews 38, 39 and references there in).

The transcription factor binding to neural-restrictive silencer (NRS), *i.e.* NRSF/REST, is a bipartite repressor possessing two distinct repression domains near the N-terminus and at the C-terminus. The transcriptional repression mechanisms via these two repression domains, *i.e.* RD-1 and RD-2, have been resolved: RD-1 recruits co-repressor mSin3 and histone deacetylase (HDAC) (40–44); whereas RD-2 binds CoREST and N-CoR (45–49). NRSF/REST also interacts with ATP-dependent chromatin remodelling SWI/SNF complex, or even basal transcriptional factor such as TATA-binding protein (TBP) (50, 51). While the overall mechanisms of NRSF/REST-dependent transcriptional regulation for cell-type specificity and other circumstances still remain to be elucidated, the

NRS–NRSF system is now well recognized to be a fundamental basis of the neuron-specific gene regulation in developing and mature neurons.

Although the transcriptional regulatory mechanism via NRS and NRSF is well understood with respect to SCG10 (superior cervical ganglia clone No.10) (3, 16, 17, 41, 52), the mechanism of cell type-specific transcriptional regulation of other members of the SCG10 gene family (53–57) remains unclear. While SCG10 seems evolved by gene duplication of the prototypic stathmin gene, evidence suggests that SCG10-like protein (SCLIP) (58) and RB3 (59), two additional members of the SCG10/stathmin family, are also expressed in neurons. Particularly, SCLIP is highly expressed in fully developed pyramidal cells and Purkinje cells, and retinal ganglion cells of the adult brain (60, 61). Unlike SCG10, SCLIP inhibits neurite outgrowth and promotes axonal branching (62, 63). SCLIP is highly expressed in mature neurons, but there is some evidence suggesting that SCLIP is also expressed in some non-neural tissues (64, 65).

To examine the factors regulating the neural-preferred transcriptional regulation of SCLIP and RB3, we isolated genomic clones containing the 5′-transcription regulatory region of these genes. The present study explored the molecular mechanisms underlying SCLIP gene expression, focusing on potential roles of NRS or NRS-like element. We identified an NRS-like sequence (NRS<sup>SCLIP</sup>) in the translation starting point in exon 1 of the SCLIP gene and showed that it was critical for the neuronal expression of the SCLIP gene. This NRS<sup>SCLIP</sup> element functioned as a cell-selective repressor wherever it was located around the SCLIP promoter; however, the level of this repressing effect varied depending upon the position relative to the promoter.

## Materials and Methods

### Library screening and determination of genomic structure

A 129 SVJ Mouse Genomic Library (Stratagene, La Jolla, CA, USA) was screened with ( $\alpha$ -<sup>32</sup>P) dCTP-labelled mouse SCLIP and RB3 cDNA probes that had been generated by reverse transcription followed by the polymerase chain reaction (RT–PCR) using primers based on the published sequence. Inserts of three independent positive lambda phage clones of the SCLIP gene were subcloned into pBluescript II SK (–) at a NotI site, and were termed pmgSCLIP-1, 2 and 3. Four positive phage clones of the RB3 gene were also cloned, and their inserts were subcloned into the same vector, and named pmgRB3-1, 2, 3 and 4. Nucleotide sequencing was performed by the use of ABI PRISM 377 (Applied Biosystems). To determine the exon–intron boundaries, we compared the genomic sequences with the cDNA sequences of mouse SCLIP and RB3 (GenBank/EMBL/DBJ: accession nos. AF069708 and AF105222).

### Primer extension assay

Primer extension was performed as described previously (16) using an inverted sequence of NRS<sup>SCLIP</sup>-containing oligonucleotide (see below) with Neuro2a RNA as a template. Extension products were separated on a 10% denaturing polyacrylamide gel with a <sup>32</sup>P-labelled size marker of Hinf-I digests of  $\Phi$ X174 DNA.

### Plasmid construction

The 5′-upstream fragments (4.7 and 1.0 kb) starting from the first methionine codon of pmgSCLIP-3 were subcloned into the luciferase reporter plasmid pGL3-Basic vector (Promega) at XhoI and NcoI sites (Luc-SL470) and HindIII and NcoI sites (Luc-SL101),

respectively. A series of deletion mutants (Luc-SL358, Luc-SL157, Luc-SL35, Luc-SL23, Luc-SL13 and Luc-SL5) were generated by digesting the Luc-SL470 with a deletion kit (TaKaRa); and Luc-dp(–) was made by deleting the 370-bp NcoI fragment of Luc-SL101. These constructs were devoid of NRS<sup>SCLIP</sup> due to the ligation to the luciferase sequence in the middle of the NRS element. For reconstitution of the NRS<sup>SCLIP</sup> sequence in the SCLIP-luciferase plasmids, Luc-NSL23, containing NRS<sup>SCLIP</sup>, was generated by altering Luc-SL23 with an oligonucleotide (5′-GGTGCTGGCCATGGTGTGGC-3′) according to the Kunkel method as described previously (41). A single NRS<sup>SCLIP</sup> element of self-complementary oligonucleotide pairs (5′-CGCCAGCACCATGGCCAGCACCG GTAC-3′ and 5′-CGGTGCTGGCCATGGTGTG CTG GCGGTAC-3′) was inserted at the most 5′-region of Luc-SL157, Luc-SL101, Luc-SL35 and Luc-SL23 at a KpnI site to generate a series of NRS<sup>SCLIP</sup>-containing constructs (Luc-NSL157, Luc-NSL101, Luc-NSL35 and Luc-NSL23).

### Transfection and reporter gene assay

RAT1, PC12, NIH-3T3 and Neuro2a cells were maintained and transfected with the aforementioned luciferase-expression plasmids driven by the SCLIP promoter by using Lipofectamine Plus (GIBCO/BRL) as described previously (41, 52). For luciferase assays, 5–8 × 10<sup>4</sup> RAT1 or PC12 cells in a 24-well plate were transfected with ~200 ng (0.06 pmol equivalent) of luciferase reporter plasmid and 50 ng of control Renilla luciferase vector (pRL-TK) (Promega), the latter used as an internal control for transfection efficiency. pBluescriptII SK(–) was used as a carrier to bring the total amounts of plasmids to 300 ng. Cellular extracts were prepared 48 h after transfection, and dual-luciferase activities were measured with a luminometer (Lummat LB96V; EG&G, Salem, MA, USA). All transfection experiments were performed three times, and averages and SEs of the luciferase activities of three independent experiments, each conducted with triplicate dishes, were calculated.

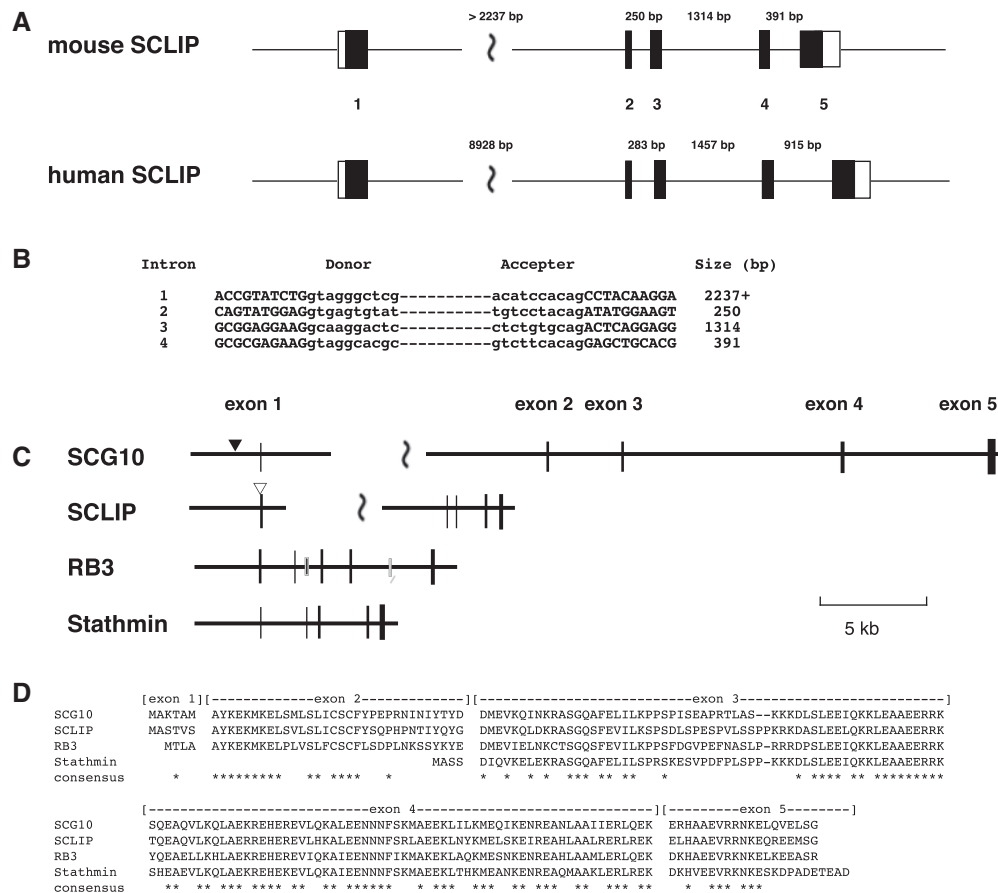
### Electrophoretic mobility shift assay

Nuclear extracts were prepared from RAT1 and PC12 cells as described previously (41, 52). Self-complementary oligonucleotide pairs were labelled with ( $\alpha$ -<sup>32</sup>P) dCTP by use of a Megaprime DNA labelling kit (Pharmacia) and purified with a QIAquick Gel Extraction Kit 250 (QIAGEN). The oligonucleotides for the gel shift assays were as follow: for the SCLIP NRS, 5′-AGCCGCCGCCAGCACCATGGCCAGCACC-3′ and 5′-AGATACGGTGTGGCCATGGTGTGGC-3′; and for the SCG10 NRS, 5′-AAGCCATTT CAGCACCACGG AGAGTGCC-3′ and 5′-AGCAGAGGCACTC TCCGTGGTGTGAA-3′. For DNA binding assays, 5  $\mu$ g of nuclear extract was incubated at room temperature for 20 min with 50,000 cpm of the labelled probes, 4  $\mu$ l of 5× binding buffer [50 mM HEPES (pH 7.9), 250 mM KCl, 25 mM MgCl<sub>2</sub>, 50% Glycerol, 25 mM DTT, 3.5 mM PMSF, 250  $\mu$ M ZnCl<sub>2</sub>], and 1  $\mu$ g of poly(dI-dC) in a total volume of 20  $\mu$ l. For supershift experiments, monoclonal antibody raised against NRSF (3B7) was used as described previously (33). The binding reaction mixtures were electrophoresed at 10 mA for 90 min at 4°C on a 4% polyacrylamide gel in modified 0.25× TEA (40 mM Tris–HCl pH 7.8, 37 mM sodium acetate) containing 2.5% glycerol. The gels were dried and exposed to an X-ray film with an intensifying screen at –80°C.

## Results

### Homology of the genomic organization of the SCLIP gene with that of other members of the SCG10 gene family

The mouse SCLIP gene was obtained by screening a liver genomic DNA library. The full-length SCLIP gene corresponding to the five coding exons was determined, and the genomic organization is presented in Fig. 1. Although the first intron sequence was not fully determined, the total size of the mouse SCLIP gene was estimated to be ~11 kb based on restriction digests (Fig. 1A). The genome sequence was compared with the previously published cDNA sequence (54, 58) in order to determine the exon–intron boundaries (Fig. 1B).



**Fig. 1** Genomic organization of the SCLIP gene and its comparison to other members of the SCG10 family. (A) Genomic organization of the mouse and human SCLIP genes. The white and black boxes indicate non-coding and coding regions, respectively. Exon numbers 1–5 of mouse and human SCLIP genes are given. (B) The exon–intron boundaries were determined by comparing mouse genomic and cDNA sequences. Intron size and the donor–acceptor splice sites of the mouse SCLIP gene are shown. Intron sequences are shown in lower case, and the donor and acceptor sites are underlined. (C) Comparison of the size and intron–exon structures of the mouse SCG10-related gene family. The genomic organization of the mouse SCLIP and RB3 genes as well as that of the mouse SCG10 and stathmin ones is presented for comparison. Interruption in the first intron of SCG10 and SCLIP genes indicates that the regions have not been fully determined for actual distance. The closed triangle in the promoter region of the SCG10 gene indicates the position of NRS, and the open triangle on the SCLIP gene denotes the position of the NRS-like sequence, NRS<sup>SCLIP</sup>. (D) Exon–domain structure relationship. The mouse SCLIP, RB3, SCG10 and stathmin genes are aligned with space introduced between exons: conserved amino acids are indicated by asterisks.

We also screened for the mouse RB3 gene, and found that it spanned 7 kb of genomic DNA and was also composed of five coding exons with 567 bp of open reading frame (ORF) covering the initiation methionine to the stop codon. Its genomic and cDNA sequences were likewise compared to determine exon–intron boundaries. Beside the five conserved exon/intron boundaries, two additional donor/acceptor sites for splicing were also found between exons 2 and 3 and between exons 4 and 5 in the mouse RB3 gene, which presumably give rise to the alternative splicing forms known as RB3' and RB3'', respectively (54, 56).

As previously reported (53), the total size of the SCG10 gene was quite different from that of the stathmin gene, even though the two genes shared a similar exon–intron structure. When the relative distances among these exons of SCG10 and the three SCG10-related genes were compared, it became clear that exons 2 and 3 were located relatively close to each other in all family member, as were exons 4 and 5,

except in the case of the RB3 gene (Fig. 1C). As aforementioned, the RB3 gene contained two additional exons, 2' and 4' used for alternative splicing. Comparison of the mouse amino acid sequences of each of the major coding exons of the SCG10 family members is presented in Fig. 1D. The homology in the mouse amino acid sequences and exon organization clearly demonstrate that all SCG10 family members were generated by gene duplication, which is consistent with the previous notion obtained from the comparison of rat RB3 and mouse SCLIP genes (58).

#### Distinct promoter feature of the SCLIP gene

Although the exon organization was conserved among the SCG10 family members, the promoter upstream sequence of the mouse SCLIP gene, at least up to 2.0 kb in length, was unrelated to that of neither SCG10 or RB3 genes (by FASTA alignment, data not shown). Even though SCLIP gene transcripts could be detected in some non-neural tissues in human (64), the expression of both SCG10 and SCLIP genes is

known to be highly abundant in neurons (58, 61). We then compared the 5'-upstream of the mouse SCLIP gene with that of the human SCLIP gene deposited in GenBank (accession number AF217796) including the first exon, and found that there was a high degree of similarity in the proximal region of the promoter between mouse and human SCLIP genes (Fig. 2A). The sequence of the first exon and splice junction were highly conserved, and the proximal promoter regions contained putative binding sites for transcriptional factors SP1 and TFII-I (Fig. 2A). However, further upstream sequence, at least for 2.0 kb, revealed little homology between the two species. To determine the transcriptional start sites of the mouse SCLIP gene, we performed a primary extension assay with Neuro2a cell-derived RNA as a template (Fig. 2B). There were at least six primary extension products suggesting that there may exist multiple transcriptional initiation sites (Fig. 2A). We conclude that this region contains the promoter of the mouse SCLIP gene. The mouse sequence was deposited to GenBank through DDBJ/SAKURA with the entry ID number: 20010723133031.31621.

To examine whether the DNA sequence upstream of exon 1 in the mouse SCLIP gene had promoter activity, we prepared a series of deletion constructs containing the first methionine codon along with various lengths of the 5'-flanking sequence from the mouse SCLIP gene, and examined their activity in both neural (PC12) and non-neural (RAT1) cells. The six longer constructs containing as few as 240 bp and as many as 4700 bp upstream from the first methionine showed significant promoter activity (Fig. 2B). Luc-SL23 was the most active promoter construct in PC12 and RAT1 cells; however, further deletion resulted in a dramatic reduction of the promoter activity (see Luc-SL13 and SL5), even though the shorter constructs still retained some transcriptional activity. The reduction in activity between Luc-SL23 and Luc-SL13 indicated the existence of an activator or the core promoter within the first 240-bp DNA sequence upstream of the first methionine. The fact that reporter constructs containing the SCLIP promoter, at least up to 4.7 kb, were equally active in neural (PC12) and non-neural (RAT1) cells indicated that cell type-specific expression required some other region(s) of the gene.

#### **Identification of an NRS-like element (NRS<sup>SCLIP</sup>) in the downstream region of the promoter of the SCLIP gene**

Surprisingly, however, we noted a sequence at the translation starting point that was similar to the NRS sequence. Independent analysis by Curmi and his colleagues identified this element in the human SCLIP gene as SCLIP6 element (64). This sequence (NRS<sup>SCLIP</sup>) was completely conserved in the mouse and human genes (Fig. 2A), but the 3'-half of the sequence was destroyed in the SCLIP-luciferase reporter constructs used in the above promoter assays (Fig. 2C). To assess whether the NRS<sup>SCLIP</sup> at the first methionine codon of the SCLIP gene plays a role in the cell type-specific expression of the SCLIP

promoter, we prepared two SCLIP promoter constructs, one containing (Luc-SL-N23) and one lacking (Luc-SL23) the NRS<sup>SCLIP</sup>, and examined their activity in cellular transfection experiments. The Luc-SL-N23 SCLIP promoter construct was prepared by mutating the first five bases 5' of the coding exon of the luciferase gene connected to the most active SCLIP promoter, Luc-SL23. PC12 and RAT1 cells were transfected with these constructs and tested for luciferase activity (Fig. 3A). The presence of NRS<sup>SCLIP</sup> led to a nearly 100-fold reduction in SCLIP promoter activity in RAT1 cells, whereas in PC12 cells only a 4-fold reduction was observed (Fig. 3A). These data indicate that the NRS<sup>SCLIP</sup> plays a major role repressing SCLIP promoter activity preferentially in RAT1 cells, suggesting that NRS<sup>SCLIP</sup> is a cell-type specific or preferred transcriptional element.

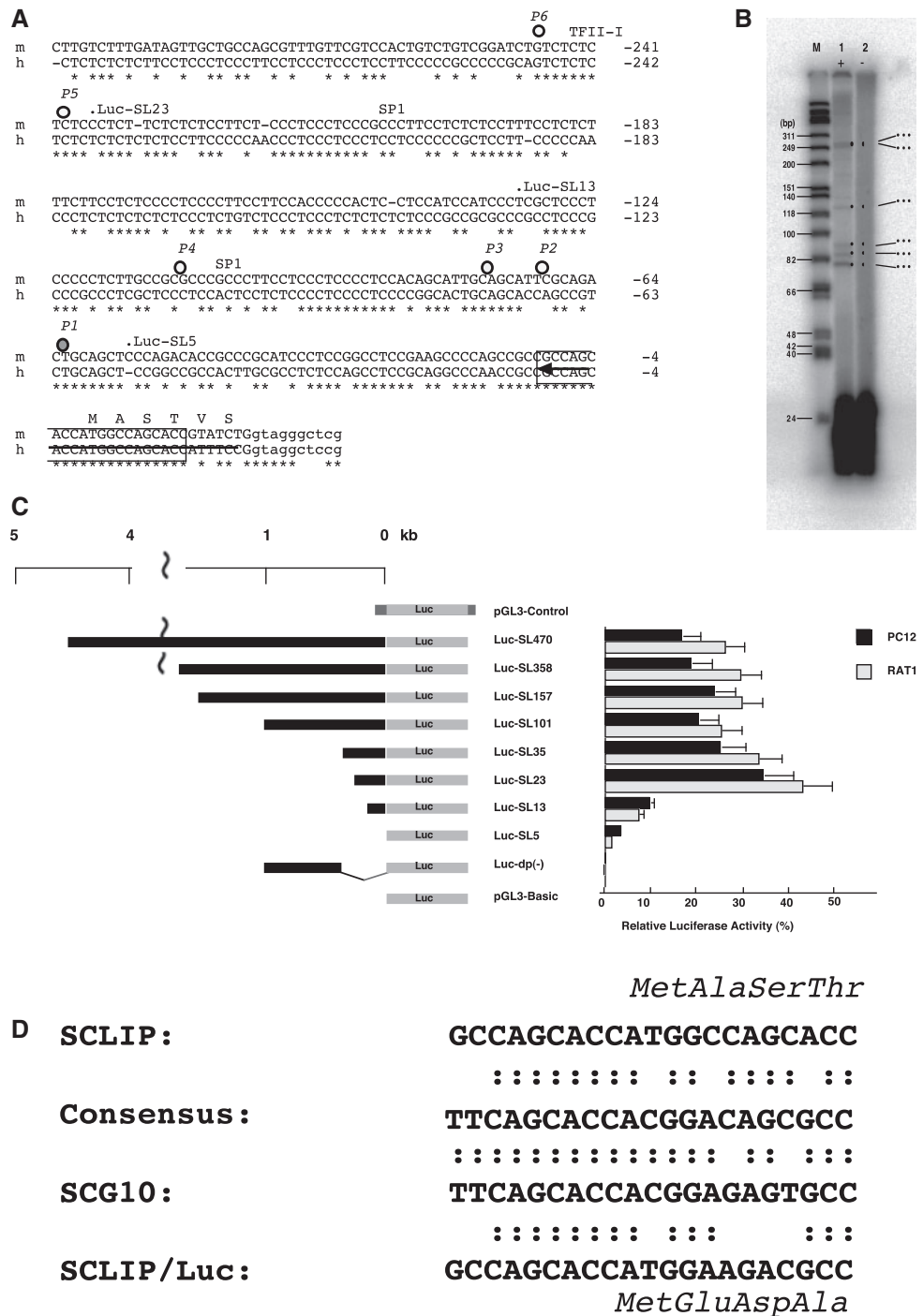
#### **Position-dependent effect of NRS<sup>SCLIP</sup>**

Since the location of NRS<sup>SCLIP</sup> at the translational initiation site at the first exon was unusual compared with that of other currently known NRS elements, we questioned if NRS<sup>SCLIP</sup> may function when it was placed in other locations. To this end, we constructed four SCLIP promoter constructs containing NRS<sup>SCLIP</sup> at the 5'-end of Luc-SL157 through Luc-SL23, respectively, and compared their activity with that of Luc-SL23 in cellular transfection experiments (Fig. 3B). As shown in Fig. 3C, the silencing effect of NRS<sup>SCLIP</sup> increased as NRS<sup>SCLIP</sup> was moved approximately -1500-bp upstream or 300-bp downstream in the SCLIP promoter region.

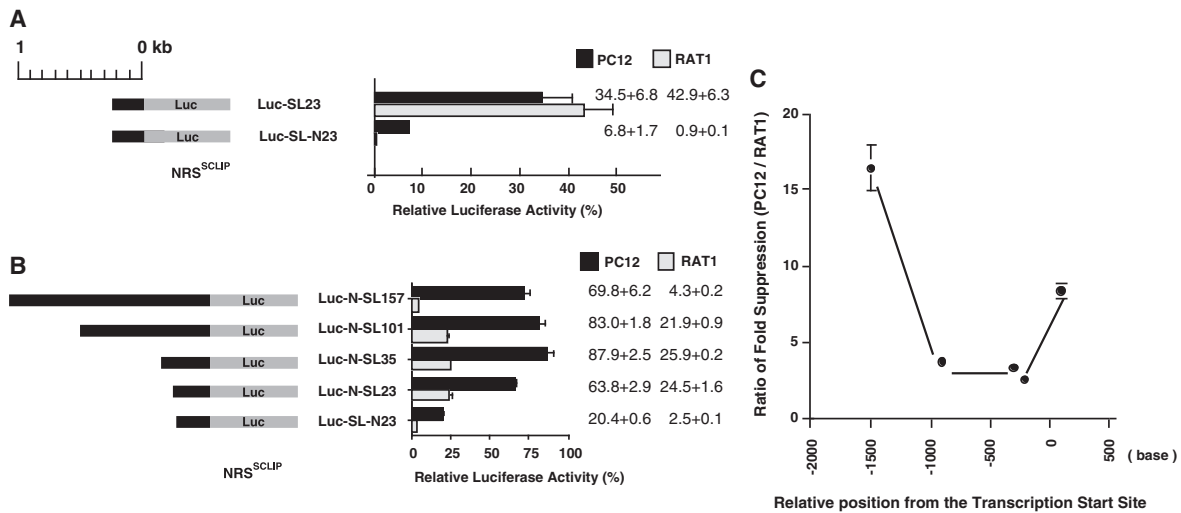
#### **NRS<sup>SCLIP</sup> is regulated by NRSF**

To learn whether the NRS<sup>SCLIP</sup> interacts with a cellular protein, particularly with NRSF, we performed an electrophoretic mobility shift assay with nuclear extracts from HeLa cells and an oligonucleotide containing a single copy of the NRS<sup>SCLIP</sup> sequence as a probe. As a confirmation of a previous report (17), binding experiments using the SCG10 NRS as a probe detected a clear-cut DNA-NRSF complex with nuclear extracts prepared from HeLa cells (Fig. 4, lane 3), and a similar gel shift of DNA-NRSF complex was detected by using the SCLIP NRS<sup>SCLIP</sup> region as a probe (Fig. 4, lane 4). The specificity of the gel shift obtained with the SCLIP probe was tested by competition experiments. Specific competition for the shift was obtained with a 10- to 100-fold molar excess of non-radioisotope-labelled SCLIP probe (Fig. 4, lanes 5–7). Similar competition was observed even with central GG/TT mutation, but was not observed with non-related sequences such as AP1 or SP1 (data not shown). Furthermore, when monoclonal anti-NRSF antibody (3B7) was included in the binding reaction, the band was supershifted, suggesting that NRS<sup>SCLIP</sup> was indeed bound by NRSF (Fig. 4, lanes 8 and 9). The supershift was not observed with pre-immune serum.

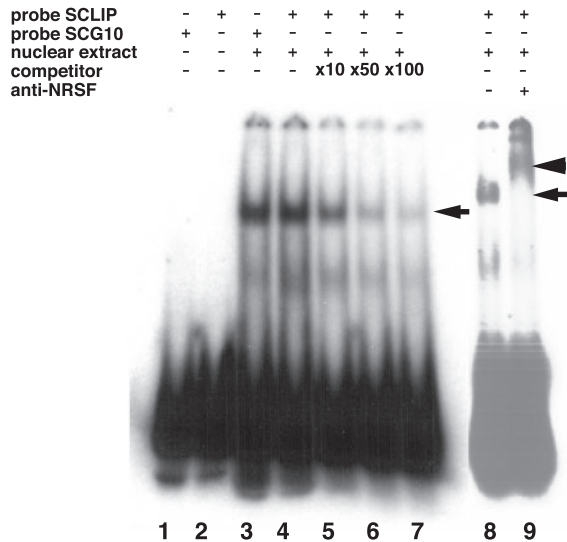
To demonstrate that the NRS<sup>SCLIP</sup> was a target for repression via NRSF, we performed cellular co-transfection experiments with NRSF expression constructs. First, a plasmid directing the expression of a



**Fig. 2** Identification of  $\text{NRS}^{\text{SCLIP}}$  in the promoter of mouse and human SCLIP genes and its effect on cell-selective gene transcription. (A) Comparison of the sequences at the 5'-end of the mouse (m) and human (h) SCLIP genes. A putative binding site for NRSF is boxed, Sp1 and TFI sites are underlined, and conserved nucleotides are marked by asterisks. First intron sequences are indicated by lower case letters, and the intron donor sequences are underlined. Transcriptional start sites (P1–P6) as determined in panel B are marked with closed circles; note that P1 is the major transcriptional initiation site. A block arrow represents the primer used in the primer extension assay experiment (see panel B). Endpoints of the deletion constructs used in panel C are indicated above the sequence. (B) Determination of transcription initiation sites of the mouse SCLIP gene by primary extension assay. Primer extension products, P1 through P6, are marked in lane 1 that was performed in the presence of RNA template. No product was obtained in the reaction without RNA template, as shown in lane 2. Size markers are  $^{32}\text{P}$ -labelled *HinfI*-digest of  $\Phi\text{X174}$  DNA. (C) Activity of SCLIP promoter constructs in cellular transfection experiments using PC12 and RAT1 cells. The relative luciferase activities of SCLIP constructs are expressed as a percentage of the SV40 promoter activity of the pGL3 control vector. The values for the pGL3 control and pGL3 basic were set at 100 and 0%, respectively. The data shown are the means  $\pm$  SE of three separate transfection experiments. (D) Comparison of  $\text{NRS}^{\text{SCLIP}}$  with the NRS consensus and  $\text{NRS}^{\text{SCG10}}$  and the boundary sequence generated in the SCLIP promoter-driven luciferase reporter gene constructs. Note that the 3'-half of the SCLIP/Luciferase plasmid is diverged extensively from the authentic NRS consensus sequence.



**Fig. 3** Characterization of NRS<sup>SCLIP</sup> with respect to cell-type selective transcriptional repression. (A) Activity of SCLIP promoter constructs with or without NRS-like sequence (NRS<sup>SCLIP</sup>) in cellular transfection experiments using PC12 and RAT1 cells. The relative luciferase activity of SCLIP constructs is expressed as a percentage of SV40 promoter activity produced by the pGL3 control vector. The values for the pGL3 control and pGL3 basic were set at 100 and 0% as in Fig. 2. The data shown are the means ± SE of three separate experiments. (B) NRS<sup>SCLIP</sup> mediates transcriptional repression of the SCLIP promoter construct position dependently in cellular transfection experiments using PC12 and RAT1 cells. The relative luciferase activity of NRS<sup>SCLIP</sup>-containing SCLIP constructs is expressed as a percentage of the activity of NRS<sup>SCLIP</sup>-lacking SCLIP constructs, the values of which were set at 100%. The data shown are the means ± SE of three separate experiments. (C) Suppression by NRS<sup>SCLIP</sup>-containing SCLIP promoter constructs is stronger in RAT1 cells than in PC12 cells. Fold-suppression was calculated for each construct in each cell line as the reciprocal of the activity relative to that of the NRS<sup>SCLIP</sup>-lacking SCLIP promoter construct. The ratio of this value for PC12 and RAT1 cells was then calculated.



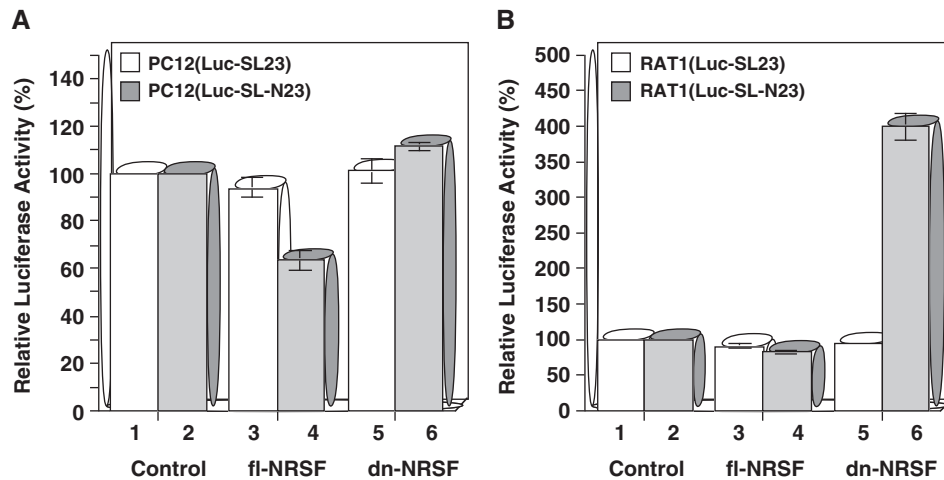
**Fig. 4** Identification of an NRS<sup>SCLIP</sup>-binding factor in HeLa nuclear extracts. Lanes 1 and 2 contained probe only; and lanes 3 and 4, probe with the nuclear extract. Lanes 5, 6 and 7 included either a 10-, 50- or 100-fold molar excess of unlabelled probe of NRS<sup>SCLIP</sup>. Lane 3 shows the NRS–NRSF complex formed with the SCG10 probe, and lane 4, a similar complex with the SCLIP probe. The arrow indicates that the complex could be competed with competitor fragments based on the NRS<sup>SCLIP</sup> sequence. Lanes 8 and 9 are supershift experiment using NRSF-specific monoclonal antibody, 3B7.

dominant-negative form of NRSF protein (dn-NRSF), which contained the internal domain but not the two repression domains within the N- and C-terminal regions, was used to transfect PC12 or RAT1 cells along with either Luc-SL23 or Luc-SL-N23 to examine whether the dn-NRSF could prevent repression of

the SCLIP promoter activity. The dn-NRSF apparently released repression of Luc-SL-N23 in RAT1 cells but not in PC12 cells (Fig. 5A and B). Second, PC12 or RAT1 cells were co-transfected with Luc-SL23 or Luc-SL-N23 and a plasmid expressing the full-length NRSF protein (fl-NRSF) to determine whether ectopic expression of NRSF in cells that normally contain low levels of NRSF activity could reduce the activity from Luc-SL-N23. Expression of fl-NRSF in PC12 cells led to repression of the activity of Luc-SL-N23 but not in RAT1 cells (Fig. 5A and B). Note that transfection experiments with Luc-SL23 reporter that does not contain NRS<sup>SCLIP</sup> did not effect luciferase activity in any condition. These results reflect the fact that the cell type-specific transcriptional regulation of the SCLIP gene is under the influence of the NRS<sup>SCLIP</sup> via NRSF.

## Discussion

The present studies provide evidence that the neuron-specific expression of the SCLIP gene is regulated, at least in part, by NRS<sup>SCLIP</sup> located in the downstream region of the promoter of the SCLIP gene. The cell-selective silencing activity of NRS<sup>SCLIP</sup> became more apparent when the element was placed far upstream, *e.g.* over 1.5kb-upstream, in the promoter region. Interestingly, when the element was placed <1kb from the core promoter, the neural-restrictive silencing activity, *i.e.* relative silencing in non-neuronal versus neuronal cells, was reduced. This finding is similar to the data showing that the NRS present in the  $\beta 2$  subunit gene of the nicotinic acetylcholine receptor was position dependent, and further even functioned as a



**Fig. 5** Effects of exogenously added full-length (fl) or dominant negative (dn) NRSF on the NRS<sup>SCLIP</sup>-bearing promoter activity. (A) Relative luciferase activities of extracts of PC12 cells transfected with reporter plasmid with or without NRSF plasmids. Lane 1, reporter (either Luc-SL23 or Luc-SL-N23) only; lane 2, reporter with fl-NRSF; lane 3, reporter with dn-NRSF. (B) Relative luciferase activities of extracts of Rat1 cells transfected with reporter with or without NRSF plasmids. Lane 1, reporter only; lane 2, reporter with fl-NRSF; lane 3, reporter with dn-NRSF.

neural preferred enhancer when it was located very close to the TATA box (34).

The reduction of silencing activity at promoter-proximal region could be due to steric hindrance of chromatin. The fact that NRSF/REST recruits mSin3 (40–42) and/or CoREST (45) suggests that NRS-dependent transcriptional repression involves chromatin modification through the recruitment of either HDAC 1/2 (40–42) or other classes of HDACs (43, 47, 48), respectively. NRS, therefore, seems to be more effective when it is located in the upstream of the promoter or in the first intron. Indeed, the most well-characterized NRSs, in sodium channel type II and SCG10 genes, were located in 1- and 1.5-kb upstream of each promoter; and other NRSs, in the brain-derived neurotrophic factor (BDNF) and the neuro-glial cell adhesion molecule L1/Ng-CAM, were found in the first intron (35–37). Since the transcriptional repression complex including NRSF/REST becomes a part of a large protein complex including the nucleosome remodelling and deacetylating (NRD, also named NURD, NuRD) complex (47, 48), it seems to require some distance of DNA looping in order to maintain an efficient silencing of the core promoter.

It seems obvious that NRS<sup>SCLIP</sup> is regulated by NRSF, as it was bound by or at least included in the complex containing NRSF (Fig. 4) and its transcriptional activity was affected by the exogenously added active or inactive NRSF constructs (Fig. 5). In non-neuronal cells, such as RAT1 fibroblasts, exogenously introduced dn-NRSF apparently de-repressed NRS<sup>SCLIP</sup>-containing Luc-SL-N23 reporter activity (Fig. 5B); however, fl-NRSF did not affect the luciferase activity of the Luc-SL-N23 construct in RAT1 fibroblast, but did affect its activity in PC12 cells. These results suggest that the expression level of endogenous NRSF is saturated in RAT1, whereas in PC12 cells NRSF is not expressed, or limited, but some functionally defective alternatively spliced forms of NRSF, such as REST4 (31, 37), is expressed.

In this context, the activity of NRS<sup>SCLIP</sup> may not be efficient as that of other NRS elements thus far found in various neuron-specific genes. It may also be possible that NRS<sup>SCLIP</sup> functions in a species specific manner as the 5'-region of the NRS homology is diverged between the human and rodent sequences (Fig. 2A). We have searched related DNA sequences in the current human genome database, but were unable to find any closely related to NRS<sup>SCLIP</sup> except other NRS-like sequences (Izumi, N. and Mori, N., data not shown). Therefore, other *cis*-regulatory elements may contribute to the neuron-specific gene expression of the SCLIP gene.

It was rather surprising to us to find little homology in promoter sequences of the three neuron-specific SCG10 family genes, *i.e.* SCLIP, RB3 and SCG10, even though the coding exons among these genes were highly homologous with each other in the human and rodent genomes. We initially anticipated that SCLIP and RB3 genes may share a similar promoter structure together with the presence of NRS for neuronal specificity; however, we were unable to find any NRS-related sequence in the promoter upstream (at least up to 3 kb) or downstream (at least up to 1 kb) of the RB3 gene. A NRS-like element was identified in the 10-kb downstream of the human RB3 gene by Curmi and his colleagues (64); however, the sequence seemed quite diverged from the authentic NRS consensus (only 13 matches out of 21 residues). Since the gene expression profiles of these SCG10-related members are distinct but similar in the sense that they are neuron-specific and developmentally regulated (60), it is still possible there may be other important regulatory elements in the genes of SCG10, RB3 and SCLIP that should differentiate the promoters of these genes from the non-cell-specific constitutive promoter of the stathmin gene.

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None declared.

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