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The helix-loop-helix transcription factor SEF-2 regulates the activity of a novel initiator element in the promoter of the human somatostatin receptor II gene

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The effects of somatostatin hormones are mediated by a family of five different seven-helix transmembrane spanning receptors (SSTR1-5). The expression of the five different SSTR subtypes displays a complex temporal- and tissue-specific pattern. To investigate the molecular mechanisms controlling the different expression patterns of the SSTRs, we cloned the 5′-flanking region of the human *SSTR2* gene. Characterization of the SSTR2 promoter resulted in the identification of a novel initiator element (SSTR2*inr*). Transcriptional activity of the SSTR2*inr* is dependent on the presence of a binding site (E-box) for basic helix-loop-helix (bHLH) transcription factors. By screening a mouse brain cDNA expression library we isolated a cDNA coding for the bHLH transcription factor SEF-2. SEF-2 binds to the E-box present in the SSTR2*inr*, both *in vitro* and *in vivo* and activates transcription from the SSTR2*inr*. A single point mutation within the E-box eliminates binding of SEF-2 and results in a complete loss of transcriptional activity of the SSTR2*inr*. Furthermore, DNA binding studies demonstrate that the basal transcription factor TFIIB can be tethered to the SSTR2*inr* through physical interaction with SEF-2. In summary, the SSTR2*inr* represents a novel type of initiator element that confers gene expression in the absence of a TATA-box or binding sites for other known initiator factors, like YY-1 or USF.

Keywords: bHLH transcription factors/initiator sequences/protein-protein interaction/somatostatin receptor/transcriptional regulation

Introduction

The pleomorphic effects of the peptide hormone somatostatin are mediated by at least five different receptors, SSTR1-5 (Bruno *et al.*, 1992; Meyerhof *et al.*, 1992; O'Carroll *et al.*, 1992; Yamada *et al.*, 1992; Yasuda *et al.*, 1992; Rohrer *et al.*, 1993), which belong to the family of seven-helix transmembrane spanning receptors. Signalling of all five receptors involves inhibition of plasmalemmal adenylate cyclase which regulates K^+ and Ca^{2+} channels and cytoplasmatic signal transduction pathways (Patel *et al.*, 1990; Rens-Domiano and Reisine, 1992). A series of studies employing Northern blot, RT-PCR and *in situ* hybridization analysis has revealed complex gene type-specific expression patterns in many regions of the central nervous system (CNS) and peripheral tissues. The areas of intensive somatostatin receptor expression include the hypothalamic-hypophyseal system, which regulates the adenohypophyseal release of GH, TSH and prolactin (Brazeau *et al.*, 1972), the widespread somatostatinergic system in the CNS modulating many cognitive and vegetative functions (Epelbaum *et al.*, 1994 and references therein) and the gastroenteropancreatic system of neuroendocrine cells that regulates gastric acid and gastroenteropancreatic hormone release (Nelson-Piercy *et al.*, 1994). Furthermore, a recent study revealed temporally and spatially regulated expression patterns of SSTR2 and SSTR4 during embryonic development of the brain. These results suggest that somatostatin signalling plays a fundamental role during neurogenesis in vertebrates (Maubert *et al.*, 1994). However, the molecular mechanisms controlling *SSTR2* gene transcription in neural cells are unknown.

Previous studies of eukaryotic class II gene promoters have shown that they can be dissected into a basal core promoter (frequently containing a TATA-box) and distal enhancer elements. The TATA box is bound by TATA-binding protein (TBP) and TFIIA, a protein that stabilizes TBP-TATA interactions, and then either promotes pre-initiation complex assembly (reviewed in Tjian and Maniatis, 1994) or alternatively recruits a large multi-protein complex termed the pol II holoenzyme (Koleske and Young, 1995; Chao *et al.*, 1996). The pol II holoenzyme in yeast represents a preassembled complex of RNA polymerase II, a set of basal transcription factors (TFIIB, TFIIE, TFIIF, TFIIH), the chromatin disrupting activity swi/snf and additional proteins necessary to activate transcription, including srb4 and srb6 (reviewed in Struhl, 1996). A key step in recruitment of the pol II holoenzyme to the core promoter is a physical interaction of TBP and TFIIB. In addition, it has been shown that enhancer binding proteins target TBP bound to the core promoter through interactions with TBP-associated factors (TAFs) and thereby support recruitment of pol II (Verrijzer *et al.*, 1995).

Frequently basal core promoters contain initiator elements displaying similarities to the sequence PyPyCANT/APyPyPy which may be necessary to direct correct transcription initiation (Smale and Baltimore, 1989). In the case of many TATA-less promoters including terminal deoxynucleotidyltransferase (TdT), human leukocyte-interferon (LeIF-j) and the viral P5 promoter, the initiator is essential for both the core promoter strength and for

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the determination of the actual transcription initiation sites. Binding of the *Krüppel*-related zinc-finger protein YY1 to the initiator has been suggested as an alternate pathway of directing initiation (Seto *et al.*, 1991; Usheva and Shenk, 1994). Similar to the TBP–TFIIA complex, YY-1 is supposed to recruit pol II by a direct physical interaction with TFIIB. Furthermore, another initiator binding protein, TFII-I, has been described which indicates that a number of alternate pathways for transcription initiation may exist (Roy *et al.*, 1993a,b).

Sequence comparison of the 5′-flanking regions in the SSTR1–5 genes demonstrates that all of these promoters lack TATA boxes and show features similar to highly regulated gene promoters like growth factor receptors and proto-oncogenes (Greenwood *et al.*, 1994; Hauser *et al.*, 1994; Xu *et al.*, 1995). To identify *cis*-regulatory promoter elements controlling SSTR2 mRNA transcription we have analysed the 5′-flanking region of the human *SSTR2* gene. Our data demonstrate that almost the entire promoter activity is mediated by a 38bp DNA sequence surrounding the mRNA initiation site. This minimal SSTR2 promoter fragment (SSTR2*inr*) confers high levels of gene expression in neuroblastoma cell lines. The SSTR2*inr* functions in the absence of binding sites for TBP or YY-1 but requires an intact E-box element. We provide evidence that the bHLH protein SEF-2 binds to and activates specifically the SSTR2*inr*. DNA binding studies reveal that SEF-2 bound to the *inr* DNA interacts physically with the basal transcription factor TFIIB. These results suggest that components of the basal transcription machinery might be tethered to the SSTR2*inr* through physical interaction with SEF-2. In summary, the SSTR2*inr* represents a novel type of initiator element that confers gene expression in the absence of a TATA-box or binding sites for other known initiator factors.

Results

Structure and function of the human SSTR2 promoter

To isolate the 5′-flanking region of the human *SSTR2* gene we screened a human genomic library with a probe representing the entire hSSTR2 coding region. A recombinant phage containing 18 kb of the *hSSTR2* genomic locus was identified and used for further analysis. The nucleotide sequence of the first 196 bp of the hSSTR2 5′-flanking region is shown in Figure 1A. Inspection of the hSSTR2 promoter revealed no TATA-like sequence. Interestingly, several potential *cis*-regulatory elements including an E-box element are clustered around 90 bp upstream of the ATG start codon (Figure 1A). To identify the site of transcription initiation, we isolated RNA from

Fig. 1. Nucleotide sequence of the first 196 bp of the hSSTR2 5′-flanking region. (**A**) The boxed areas represent the T-rich sequence and the hSSTR2 E-box. The two arrows indicate the transcription initiation sites. The ATG start codon of the hSSTR2 coding region is underlined. (**B**) Identification of the site of transcription initiation by RNase protection assay. The antisense RNA used to map the 5′-end of the hSSTR2 mRNA is a 357 base transcript (lane 6). The protected probe corresponds to a fragment of 44 bp. The RNA probe was hybridized to total RNA from LAN-1 cells (lane 5), MCF-7 cells (lane 4) or NIH 3T3 cells (lane 3). As a control no RNA (lane 2) or yeast tRNA (lane 1) were used. The major protected fragment is indicated by an arrow.

the human cell lines, LAN-1 and MCF-7. LAN-1 cells were used because these cells express the endogenous hSSTR2 gene (Manil *et al.*, 1994). RNase protection assays demonstrate that the major hSSTR2 mRNA initiates 6 bp downstream of the E-box. A minor start site is located 2 bp further downstream (Figure 1B, lane 5). No specific signals were detected in control RNA from fission yeast or receptor negative NIH 3T3 cells (Figure 1B, lanes 1 and 3).

Next, we fused various portions of the hSSTR2 promoter to a firefly luciferase reporter gene. Transcriptional activity of the promoter constructs was monitored following transient transfection in the SSTR2 expressing human and mouse neuroblastoma cell lines LAN-1, NGP and Neuro2A (N2A) (Manil *et al.*, 1994). As a control we used NIH 3T3 and HeLa cells which do not express the *SSTR2* gene. The reporter construct –1.8 kb hSSTR2LuC supported high levels of expression in both the human and mouse neuroblastoma cell lines. In contrast, the hSSTR2LuC reporter gene was barely active in NIH 3T3 and HeLa cells (Figure 2A, lane 1). Subsequent deletions of 5′-flanking sequences did not alter the promoter activity drastically (Figure 2A, lanes 2–4). A reporter construct containing only the promoter region from position –30 bp to + 45 bp was still highly active (Figure 2A, lane 5).

To narrow down further the minimal sequences required for hSSTR2 promoter activity, we generated several additional 5′ and 3′-deletion mutants and assayed their transcriptional activity in Neuro2A and NGP cells. Deletion of 3′-sequences up to position +4 bp did not change the activity of the reporter construct significantly (Figure 2B, compare lanes 1–3). However, further 3′-deletions rendered the promoter inactive (Figure 2B, lane 4). Deletions of 5′-sequences up to position –22 bp only mildly alters the activity of the reporter gene (Figure 2B, lane 5), whereas a further 5 bp deletion is deleterious to reporter activity (Figure 2B, lane 7). Interestingly, the introducing of two point mutations (TTTTCC to TTTT<u>GG</u>) into the T-rich sequence located immediately upstream of the E-box (–20 bp to –13 bp) reduced the *inr* activity by 30% only (Figure 2B, lane 6). These results indicate that the minimal region necessary for transcriptional activity of the hSSTR2 promoter is located between nucleotide positions –22 bp and +4 bp. The obvious lack of the TATA-box or a TATA-like sequence suggested that the minimal hSSTR2 promoter may function as an initiator element on its own. In accordance with the definition of an initiator element, transcriptional activity of the hSSTR2 initiator (SSTR2*inr*) is also strictly orientation dependent (Figure 2B, lane 8). One interesting feature of the SSTR2*inr* is the presence of an E-box (CAGATG) which can serve as a binding site for basic helix–loop–helix (bHLH) transcription factors. To establish whether this E-box is contributing to the transcriptional activity of the SSTR2*inr* we generated a mutant SSTR2*inr* reporter with a single point mutation in the E-box (CA<u>**A**</u>ATG). This point mutation completely inactivates the hSSTR2*inr*, which indicates that the transcriptional activity of the *inr* is dependent on the integrity of this particular E-box (Figure 2B, lane 9).

A comparison of the hSSTR2*inr* with the Inr consensus PyPyCANT/APyPyPy raised the possibility of a putative initiator-like sequence located between the E-box and the transcription start site. To prove that this sequence is not a standard Inr or initiator-like element we introduced three point mutations into the region between –5 bp and –1 bp (TCACACT to T<u>**AT**</u>C<u>**C**</u>CT). These mutations have been shown to completely abolish standard Inr activity (Javahery *et al.*, 1994; Kaufmann and Smale, 1994). Importantly, these mutations do not inactivate the transcriptional activity of the hSSTR2*inr* (Figure 2C, compare lanes 1–4). To demonstrate further that the E-box and not the sequence between –5 bp and –1 bp (putative initiator-like sequence) is part of the hSSTR2*inr* we only inverted the orientation of the E-box which should inactivate the promoter if the putative initiator like-sequence is irrelevant. Indeed, according to the definition of an initiator element, transcriptional activity of this mutated SSTR2*inr* is absolutely dependent on the orientation of the E-box (Figure 2C, lane 5). These results suggest that the SSTR2*inr* functions in the absence of a standard initiator-like sequence but requires the intact E-box element.

To demonstrate that the luciferase reporters use the same transcription start site as the endogenous *hSSTR2* gene, we performed a series of primer extension assays. The results shown in Figure 2D reveal that the *inr* facilitates transcription initiation in the LuC reporters from the same nucleotide as in the endogenous *hSSTR2* gene (Figure 2D, lane 2). The promoterless parental luciferase plasmid as well as the mutant M6 harbouring a single point mutation in the E-box did not support transcription (Figure 2D, lanes 1 and 3). In addition, we cloned oligonucleotides containing the SSTR2*inr* or the point mutation M6 into the promoterless CAT reporter plasmid pBLCAT3 (Luckow and Schütz, 1987) and assayed whether the *inr* is able to initiate RNA transcription in a yet different promoter context. Inspection of the 5′-flanking sequence of the pBLCAT3 reporter did not reveal any cryptic TBP binding site. Again, the primer extension assays demonstrate that the SSTR2*inr* directs RNA synthesis from the same nucleotides as in the LuC reporters and the endogenous *hSSTR2* gene (data not shown). Taken together, these data indicate that the hSSTR2 promoter contains a novel type of initiator element and that the transcriptional activity of this *inr* is dependent on the presence of the intact E-box.

Isolation of SEF-2, a helix–loop–helix protein binding to the SSTR2inr

To identify and clone proteins capable of binding to the SSTR2*inr* we screened a murine brain λgt11 expression library using a tetramerized initiator as a probe. A brain cDNA library was chosen because SSTR2 is known to be widely expressed in the brain (Epelbaum *et al.*, 1994). Two independent recombinant phages were identified after screening of 2×10^5 plaques. DNA sequence analysis revealed that the cDNA inserts encode overlapping carboxy-terminal fragments of the murine homologue of the human bHLH transcription factor SEF-2 1B (Corneliussen *et al.*, 1991), which was previously characterized as immunoglobulin transcription factor ITF in B lymphocytes (Henthorn *et al.*, 1990). To obtain a full-length murine SEF-2 cDNA we used the partial clones to rescreen the brain cDNA library and isolated four independent clones. One of these clones spanning nucleotides 1–2430 contains the entire open reading frame coding

A

Construct	NGP	N2A	LAN-1	3T3	HeLa
1 (-1800 … E-Box … +45 LuC)	120	350	18	1	1
2 (-840 … +45 LuC)	160	632	27	1	1
3 (-201 … +45 LuC)	300	860	34	1	1
4 (-89 … +45 LuC)	240	610	37	1	1
5 (-30 … +45 LuC)	260	524	34	1	1
6 (LuC)	1	1	1	1	1
7 (SV 40 LuC)	400	500	40	125	200

Luciferase Activity

B

Construct	N2A	NGP
1 WT	530	250
2 Inr	550	260
3 M0	540	250
4 M1	2	2
5 M8	320	180
6 M12	225	130
7 M7	2	2
8 Rev	1	2
9 M6	1	1
10 (LuC)	1	1
11 (SV 40 LuC)	620	440

Luciferase Activity

C

Construct	N2A	NGP
1 Inr (TCACACTG)	530	250
2 M11 (TATCCCTG)	350	170
3 M8 (TCACACTG)	320	180
4 M9 (TATCCCTG)	220	210
5 M10 (E-Rev, TCACACTG)	1	1
6 (LuC)	1	1

Luciferase Activity

D

C T A G LuC INR M6

1 2 3

Fig. 2. Functional analysis of the hSSTR2 promoter. (**A**) Transient transfections of NGP, Neuro2A (N2A), LAN-1, NIH 3T3 and HeLa cells using 1 µg of the indicated reporter plasmids (lanes 1–5). As a control 1 µg of either the promoterless reporter plasmid pGL3 basic or the SV40 promoter containing pGL2Prom reporter (Promega) were used (lanes 6 and 7). The two arrows indicate the transcription initiation sites. The E-box is represented by an open rectangle. The numbers indicate positions of the respective nucleotides within the hSSTR2 promoter. (**B**) Identification of the hSSTR2*inr* element. One microgram of the indicated reporter plasmids was transfected into Neuro2A (N2A) or NGP cells (lanes 1–11). Reporter construct M6-LuC containing a single point mutation in the E-box (MUT) is indicated (lane 9). The T-rich sequence is represented by TC enclosed in a rectangle. The double point mutant M12 is symbolized by TGG enclosed in a rectangle (lane 6). (**C**) The hSSTR2 promoter contains no standard *inr*-like sequence. One microgram of the indicated reporter plasmids was transfected into Neuro2A (N2A) or NGP cells (lanes 1–6). The three point mutations introduced into the region between –5 bp and –1 bp are shadowed (Mutants M9-LuC and M11-LuC). Reporter construct M10-LuC (lane 5) containing only a reversed E-box is indicated by E-Rev enclosed in a rectangle. (**D**) Confirmation of the *inr* transcription start site in the context of the LuC reporter gene. Primer-extension analysis was performed using a primer complementary to the luciferase coding region (pGL3 luciferase reporter, Promega). The end-labelled primer was annealed to 30 µg of total RNA from Neuro2A cells transfected with 5 µg of Inr-LuC (lane 2), M6-LuC, the mutant harbouring a G to A point mutation in the E-box (lane 3) or the promoterless pGL2LuC (lane 1). The length of the extended product was established by comparison with the sequencing reactions carried out using the same primer and double stranded DNA as a template.

for 667 amino acids of the murine SEF-2 protein (Figure 3). A sequence comparison with the human SEF-2 1B protein which was cloned previously from a thymocyte cDNA library (Corneliussen *et al.*, 1991) indicates that the human and murine proteins are highly conserved. Among 667 amino acids, only 15 mostly conservative exchanges are detected and none of them are located within the bHLH domain (Figure 3). The murine SEF-2 cDNA sequence was submitted to the EMBL data library (accession No. X91753).

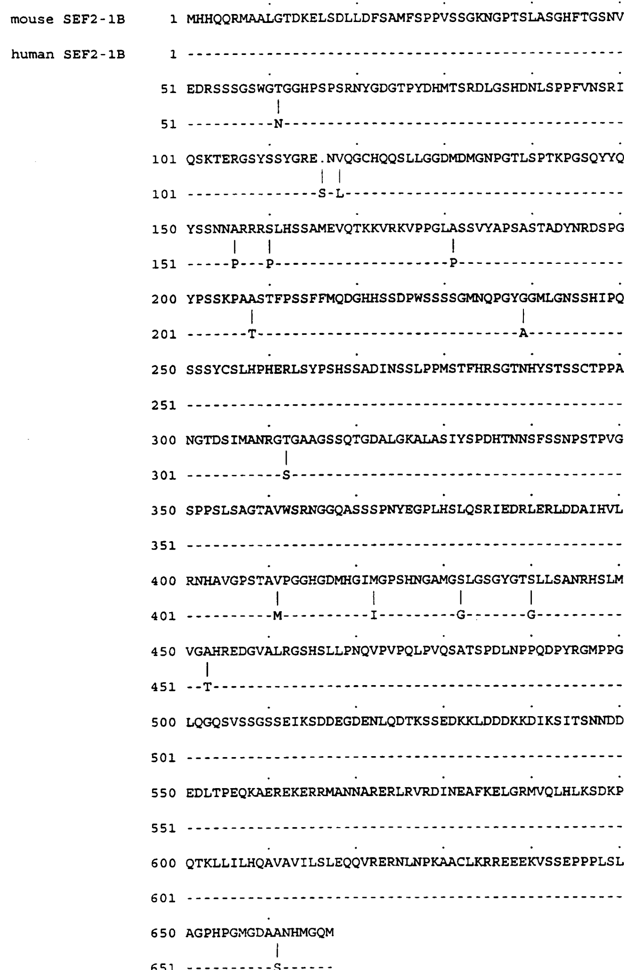
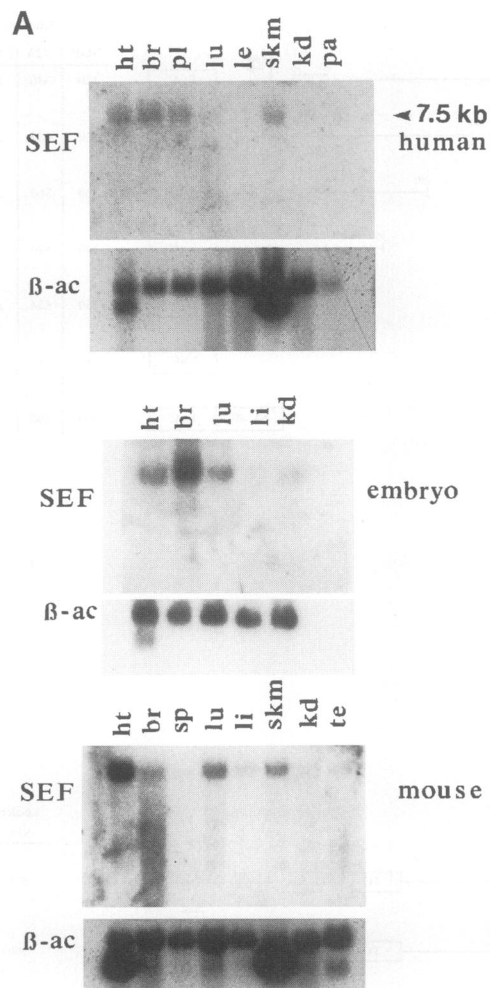


Fig. 3. Protein sequence of mouse SEF-2. Comparison of the murine SEF-2 (top) and human SEF-2 1B (bottom) protein sequence. Human amino acid residues differing from those of the murine protein are indicated below. The bHLH domain spanning residues 563–621 is entirely conserved between the two species.



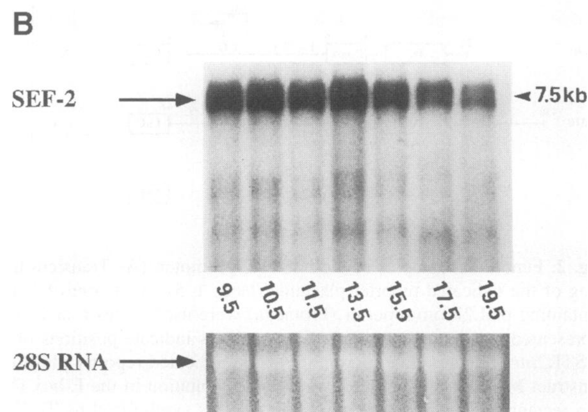


Fig. 4. Expression analysis of SEF-2. (**A**) Northern blot analysis of SEF-2 expression in adult human tissues (top), embryonic human tissues (middle) and adult murine tissues (bottom). The 7.5 kb transcripts are indicated (SEF). ht (heart), br (brain), pl (placenta), lu (lung), le (liver), skm (skeletal muscle), kd (kidney), pa (pancreas), li (liver), sp (spleen), te (testis). The β-actin (β-ac) controls are shown below each blot. (**B**) SEF-2 expression during murine embryonic development between gestational days 9.5 and 19.5 pc. The 7.5 kb transcripts are indicated by the arrow. The 28S RNA controls are shown below.

SEF-2 is widely expressed in adult organs including neural and muscle tissues and during embryonic development

To analyse the pattern of SEF-2 mRNA expression and verify that SEF-2 is expressed in tissues that also express SSTR2, like brain, lung or muscle (Rohrer *et al.*, 1993; Eppelbaum *et al.*, 1994) we hybridized Northern blots of adult and embryonic murine and human tissues with an SEF-2 cDNA probe. To avoid crossreactions with other bHLH transcription factors we used a probe lacking the carboxy-terminal bHLH domain. As shown in Figure 4A, the 7.5 kb human SEF-2 transcripts are detected in adult heart, brain, placenta, skeletal muscle and to a lesser extent in the lung. Interestingly, in developing embryonic human tissues, SEF-2 mRNA is expressed foremost in brain (Figure 4A), where also abundant expression of SSTR2 occurs during neuroblast differentiation (Epelbaum *et al.*, 1994 and our unpublished results). At lower levels, the signals are detected in embryonic human heart and lung. In adult mice SEF-2 is expressed in heart, brain, lung and skeletal muscle (Figure 4A). Hybridization of a Northern blot prepared from whole murine embryos revealed that two SEF-2 mRNAs are expressed at significant levels during all stages of development, from day 9.5 through to birth (Figure 4B). An identical pattern of SEF-2 mRNAs was detected in the two neuroblastoma cell

lines N2A and NGP but not in undifferentiated PA-1 teratocarcinoma cells (data not shown).

Sequence-specific binding of SEF-2 to the inr E-box

To study the consequences of physical interaction between the SEF-2 protein and the *inr* DNA we performed electrophoretic mobility shift assays (EMSA) with a ^{32}P-labelled oligonucleotide containing a single *inr* element and recombinant SEF-2 proteins. GST-SEF-2 fusion protein forms a retarded complex with the *inr* element (Figure 5A, lane 2). This complex is specifically competed with by a 50-fold molar excess of the unlabelled homologous *inr* oligonucleotide (Figure 5A, lane 4). The mutant M1, which represents the 5′-part of the *inr* sequence (–30 bp to –11 bp) does not compete (Figure 5A, lane 6). In contrast, the mutant M2 competes efficiently, indicating that sequences within the 3′-part of the *inr* element are sufficient for SEF-2 binding (Figure 5A, lane 8). Accordingly, the mutants, M3 and M4, which contain point mutations in the 5′-part of the *inr* compete as well as the wild-type *inr* element (Figure 5A, lanes 10 and 12). Finally, we tested the mutant oligonucleotide M5, which contains only the *inr* E-box sequence. This mutant competes as efficiently as the wild-type (Figure 5A, lanes 14). In contrast, the mutant oligonucleotide M6, which contains the single point mutation within the E-box is unable to compete (Figure 5A, lanes 16). Moreover, this E-box mutation results in a complete loss of *inr* activity in transient transfections and primer extension assays (see Figure 2B and D).

To establish further that the SSTR2*inr* binds to the bHLH transcription factor SEF-2 expressed endogenously in neuronal cells, we performed electrophoretic mobility shift assays with a ^{32}P-labelled oligonucleotide M7, containing *inr* sequences from –17 bp to +8 bp. Proteins, present in whole cell extracts from human NGP cells, are able to form a specific DNA–protein complex, C1 (Figure 5B, lane 2). The complex is competed by a 25-fold molar excess of either the unlabelled homologous oligonucleotide, M7 (Figure 5B, lane 3), or an oligonucleotide representing the entire *inr* sequence (Figure 5B, lane 7). The mutant oligonucleotide M6, which contains the single point mutation within the E-box, fails to compete (Figure 5B, lane 4). Finally, we challenged the EMSA with various antibodies directed against different bHLH transcription factors. Complex C1 is specifically supershifted by an antibody raised against human SEF-2 protein (Dako, Hamburg, Germany), which demonstrates that the bHLH transcription factor SEF-2 is part of this DNA–protein complex (Figure 5B, lane 5). Incubation with an unrelated monoclonal α-Flag control antibody has no influence on the mobility of complex C1 (Figure 5B, lane 6).

The transcriptional activity of the SSTR2inr is mediated by SEF-2

To explore the potential importance of SEF-2 for the transcriptional regulation of the SSTR2*inr* we transfected Neuro2A cells with the reporter constructs Inr-LuC and M6-LuC. Co-transfection of the SEF-2 expression plasmid results in a robust induction of Inr-LuC activity (Figure 5C, bar 2). In contrast, SEF-2 fails to activate the M6-LuC reporter which contains the single point mutation within the E-box sequence (Figure 5C, bar 5). As expected, activity of the SV40 control plasmid is not altered by SEF-2 (Figure 5C, compare bars 7 and 8). Interestingly, a SEF-2 mutant which lacks the amino-terminus (ΔNSEF) is unable to induce Inr-LuC activity (Figure 5C, bar 3). Both proteins, SEF-2 and ΔNSEF were expressed to similar levels in the cells tested (data not shown). These results suggest that the amino-terminus of SEF-2 might provide a function which is necessary for transactivation. To address the question of whether SEF-2 is able to induce SSTR*inr* activity in non-neuronal cell lines which do not express endogenous SEF-2 protein, we co-transfected SEF-2 expression plasmids into HeLa and NIH 3T3 cells and tested for their ability to induce the Inr-LuC reporter gene. As shown in Figures 5D-E, bars 1 and 2, SEF-2 fails to activate the Inr-LuC reporter plasmid in both cell lines. These results suggest that additional factors, possibly cell-specific co-activator proteins are important for SEF-2 function in neuronal cells. Taken together, the results shown in Figures 5 demonstrate that the E-box is sufficient to allow both transactivation of the SSTR*inr in vivo* and binding of the bHLH transcription factor SEF-2 *in vitro*.

SEF-2 interacts physically with components of the basal transcription machinery in vitro

To obtain initial insights into the mechanism of SEF-2 mediated initiator activity, we performed electrophoretic mobility shift assays with the ^{32}P-labelled oligonucleotide, which contains *inr* sequences from –30 bp to +8 bp. Purified, recombinant TBP, TFIIB or TFIIA proteins do not bind to this probe, which demonstrates that these proteins do not recognize the initiator element (Figure 6A, lanes 2–3). As shown before, SEF-2 forms a retarded DNA–protein complex, CI (Figure 6A, lane 4). Importantly, performing the EMSA in the presence of both SEF-2 and TFIIB proteins results in the formation of an additional complex, CII (Figure 6A, lane 5). In contrast, neither TBP nor TFIIA are able to form heteromeric complexes with the DNA-bound SEF-2 (Figure 6A, lane 6 and 7). In addition, the mutant SEF-2 protein lacking the amino-terminus (ΔNSEF) also fails to form a heteromeric DNA–protein complex with TFIIB (Figure 6B, compare lanes 2 and 3). Interestingly, ΔNSEF also fails to induce Inr-LuC activity in transfection experiments (Figure 5C, bar 3). Taken together, our data demonstrate that the basal transcription factor TFIIB can be tethered onto this initiator by forming a protein–protein complex with SEF-2 bound to DNA. The physical interaction between the two transcription factors is mediated by the amino-terminus of SEF-2. These results suggest that SEF-2 might be able to recruit the basal transcription machinery to the initiator element through direct interaction with TFIIB.

Discussion

Our results indicate that a small, 38 bp DNA element (SSTR2*inr*) located around the mRNA initiation site is necessary and sufficient for the hSSTR2 promoter to mediate high levels of SSTR2 mRNA transcription in neuronal cells. Interestingly, the SSTR2*inr* contains a T-rich sequence and an E-box. Our results suggest that the minimal sequence required for initiator function is located between positions –22 bp and –6 bp. Deletion of

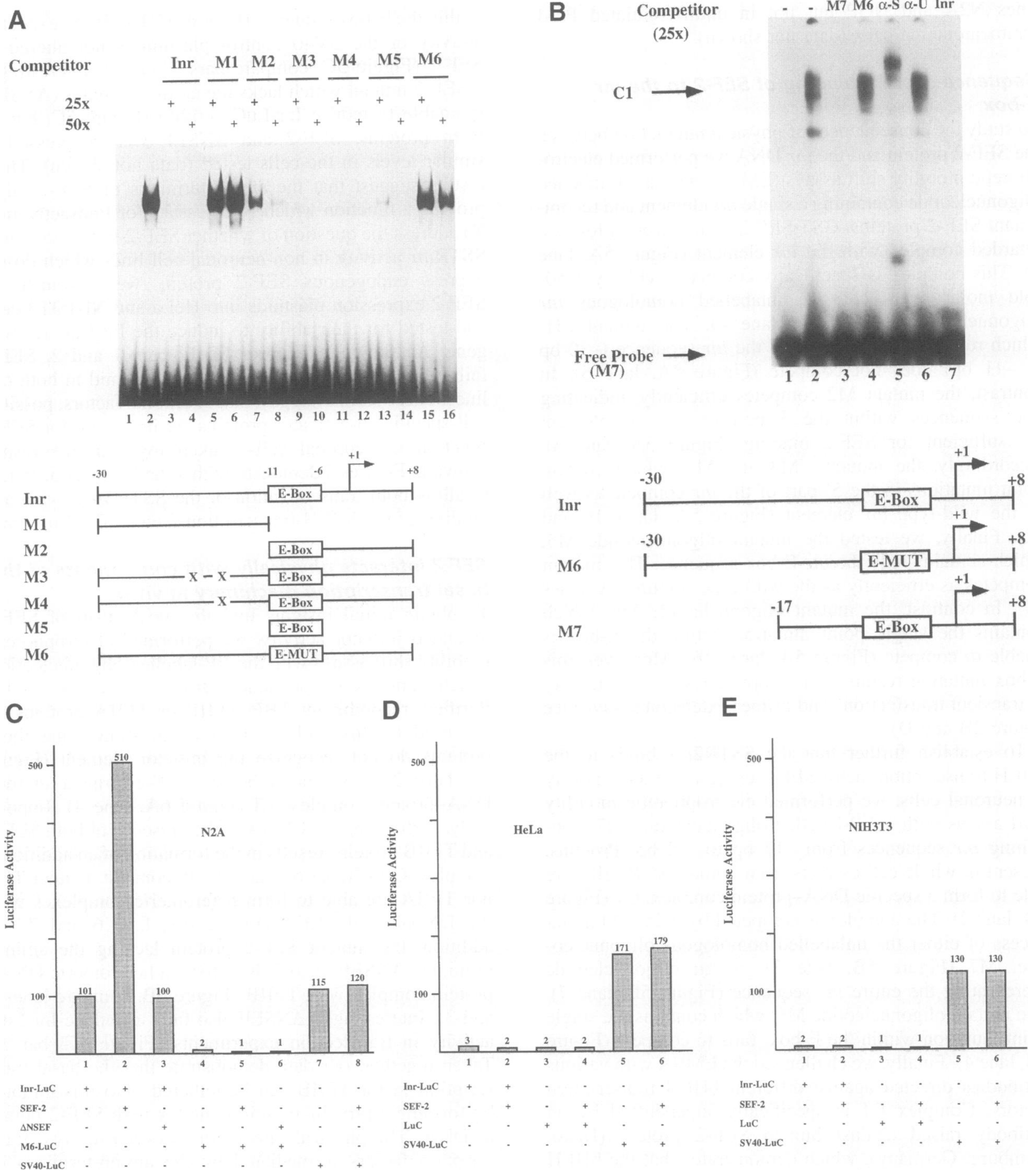


Fig. 5. Sequence specific binding of SEF-2 to the *inr* E-box *in vitro*. (**A**) The EMSAs were performed using a ^{32}P-labelled oligonucleotide containing the inr sequence from position −30 bp to +8 bp and purified recombinant SEF-2 protein (lane 2). Competition experiments were performed with either a 25- or 50-fold molar excess of the indicated competitors. The SEF-2–*inr* complex is competed by the unlabelled homologue of the *inr* oligonucleotide (lanes 3–4), the oligonucleotides M2 (lanes 7–8), M3 (lanes 9–10), M4 (lanes 11–12) and M5 (lanes 13–14). The oligonucleotide M1 representing the 5′-part of the *inr* (lanes 5–6) and the oligonucleotide M6 which contains a single point mutation in the E-box, fail to compete (lanes 15–16). The E-box and the mutated E-box (E-mut) are marked by open rectangles. Mutations in the 5′-part of the *inr* are represented by an X. The numbers indicate positions of the respective nucleotides within the hSSTR2 promoter. (**B**) SEF-2 binds to the SSTR2*inr in vivo*. Four microlitre samples of whole cell extracts prepared from human NGP cells were incubated with 1 ng of the ^{32}P-labelled oligonucleotide M7, which represents the *inr* element from position −17 bp to +8 bp (lanes 2–7). The DNA–protein complex C1 is specifically competed by a 25-fold molar excess of either oligonucleotide M7 (lane 3) or *inr* (lane 7). In contrast, oligonucleotide M6 fails to compete (lane 4). To establish the composition of the complex C1, the EMSA was challenged with specific antibodies directed against the human SEF-2 protein (lane 5), or an unrelated α-Flag control antibody (lane 6). (**C**) SEF-2 mediates transcriptional activation of the SSTR2*inr*. Transcriptional activity of 1 μg of the reporter constructs Inr-LuC (bars 1–3), M6-Luc (bars 4–6) and SV40-LuC (bars 7–8) in Neuro2A cells co-transfected as indicated (+) with 500 ng expression plasmids coding for SEF-2 or ΔNSEF. (**D–E**) SEF-2 fails to activate the SSTR2*inr* in HeLa and NIH 3T3 cells. Transcriptional activity of 1 μg of the reporter constructs Inr-LuC (bars 1–2), pGL2LuC (bars 3–4) and SV40-LuC (bars 5–6) in HeLa and NIH 3T3 cells co-transfected as indicated (+) with 500 ng of SEF-2 expression plasmids.

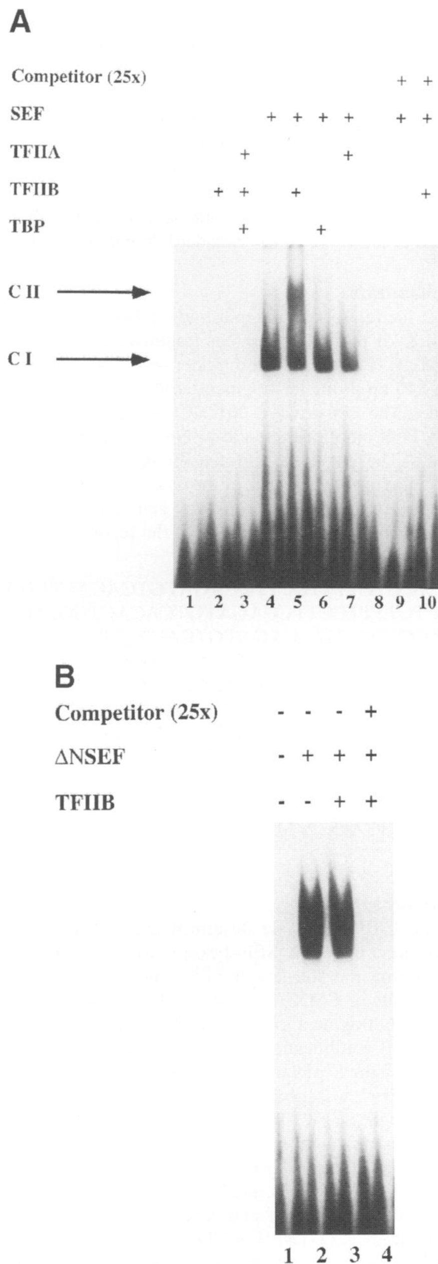


Fig. 6. (**A**) SEF-2 interacts physically with the basal transcription factor TFIIB *in vitro*. Purified recombinant TBP, TFIIB, TFIIA and SEF-2 proteins were incubated with 1 ng of ^{32}P-labelled oligonucleotide, representing the *inr* element from position –30 bp to +8 bp. Only SEF-2 is able to form a retarded DNA–protein complex CI (lane 4). To assay for a possible complex formation between SEF-2 bound to DNA and the basal transcription factors, equal amounts of TFIIB (lane 5), TBP (lane 6) or TFIIA (lane 7) were incubated with recombinant SEF-2. Only TFIIB is able to form an additional heteromeric SEF-2–TFIIB–*inr* complex CII (lane 5). (**B**) The amino-terminal deletion mutant ΔNSEF binds efficiently to the ^{32}P-labelled oligonucleotide representing the *inr* element from position –30 bp to +8 bp (lane 2), but fails to interact with recombinant TFIIB protein (lane 3). The ΔNSEF–*inr* complex is specifically competed by a 25-fold molar excess of the unlabelled *inr* oligonucleotide (lane 4).

the T-rich sequence located immediately upstream of the E-box (–20 bp to –13 bp) renders the SSTR2*inr* inactive (Figure 2B, lane 7). In contrast, introduction of two point mutations (TTTTCCTT to TTTT<u>GG</u>TT) clearly does not inactivate the SSTR2*inr* but only mildly influences *inr* activity (Figure 2B, lane 6). In principle, the T-rich sequence might serve as a transcription factor binding site important for SSTR2*inr* function. However, we are unable to detect binding of cellular proteins to the T-rich sequence so far (data not shown). One alternative interpretation of the results would be that the T-rich sequence is not a binding site for cellular proteins but rather serves as a structural motif which might allow the DNA to adopt a transcriptionally active conformation.

Although the T-rich sequence is necessary for SSTR2*inr* activity, the exact role of this sequence is not yet clear. As an initial step in the characterization of the SSTR2*inr* we therefore decided to investigate the function of the E-box first. The compact SSTR2*inr* is distinguished from other eukaryotic class II gene promoters by two features. First, it does not harbour classical initiator elements known to recruit factors of the basal transcription machinery. Second, the SSTR2*inr* activity is dependent on a functional E-box element. Simultaneously, the SSTR2*inr* matches two important criteria defining it as a genuine initiator, i.e. it functions in an orientation-dependent manner and it mediates RNA synthesis independently of other initiation elements and of its natural promoter context.

Screening of a brain cDNA expression library revealed that the bHLH transcription factor SEF-2 binds specifically to the SSTR2*inr*. Electrophoretic mobility shift analysis using purified recombinant SEF-2 protein demonstrates that SEF-2 specifically interacts with the SSTR2*inr* E-box but fails to interact with a mutant harbouring a point mutation in the E-box. Accordingly, introduction of the same point mutation into a SSTR2*inr* reporter plasmid abolishes both transcriptional activity and RNA initiation. DNA binding studies performed with crude nuclear extracts from human neuroblastoma cells demonstrate the formation of a specific *inr*–protein complex. This *inr*–protein complex is specifically recognized by an α-SEF-2 antibody, clearly demonstrating that SEF-2, present in neuronal cells, binds to the *inr*. Furthermore, co-transfection of SEF-2 expression plasmids results in a robust activation of Inr-LuC reporter gene activity in neuronal cells. In contrast, an SEF-2 mutant lacking the amino-terminus (ΔNSEF) is unable to induce Inr-LuC activity. In addition, SEF-2 fails to activate the M6-Luc reporter which contains the single point mutation in the SSTR*inr* E-box. Taken together, the results of DNA binding studies, transient transfection assays and primer extension analysis are consistent with the hypothesis that the activity of the SSTR2*inr* is dependent on binding to the bHLH protein SEF-2.

Exploring the mechanism of transcriptional activation mediated by the SSTR2*inr* E-box, we tested the interactions between SEF-2 and transcription factors known to be involved in recruiting the basal transcription machinery. EMSAs reveal that the SSTR2*inr* element alone does not bind to the basal transcription factors TBP, TFIIA, TFIIB or the initiator protein YY-1 (data not shown). However, the data presented in Figure 6 indicate that SEF-2 interacts specifically with TFIIB thereby tethering this basal transcription factor onto the initiator. Interestingly, the amino-terminal deletion mutant ΔNSEF, which is unable to induce Inr-LuC activity, also fails to interact with TFIIB. These results suggest that SEF-2 might be able to recruit the basal transcription machinery onto the SSTR2*inr* via physical interaction with TFIIB. It is tempting to speculate

that SEF-2 bound to the *inr* might perform a similar function in recruiting TFIIB, like the TATA-bound TBP–TFIIA complex. Recent studies in yeast have shown that RNA polymerase II is found in a large multiprotein complex, the pol II holoenzyme, containing TFIIB, E, F, H and other proteins necessary for enhanced transcription (Koleske and Young, 1995; Struhl, 1996). Therefore, an interaction between SEF-2 and TFIIB, as observed in our studies, suggests that SEF-2 might have the potential to recruit pol II activity to the SSTR2*inr*. Roeder and co-workers have previously defined a bHLH-dependent pathway of basal transcription initiation mediated by TFII-I (Roy *et al.*, 1991, 1993a,b). However, transcription initiation involving SEF-2 differs mechanistically from the TFII-I dependent pathway because SSTR2*inr* mediated transcription is not inhibited by c-myc and also the SSTR2*inr* E-box does not serve as a binding site for the bHLH transcription factor USF.

E-box elements with the consensus sequence CANNTG are known to interact with a large number of bHLH transcription factors including muscle-specific factors, myc, max, mad and USF. The two central nucleotides contribute to the binding specificity of each class of bHLH factor and determine the specificity of binding to a particular E-box. The point mutation M6 (see Figure 5A and B) clearly demonstrates that the interaction between SEF-2 and the SSTR2*inr* requires a G as one of the central E-box residues. Consistently, myc, max, mad and USF proteins, which bind preferentially to the sequence CACGTG (Blackwell *et al.*, 1990) fail to interact with the SSTR2*inr* in DNA binding studies (Blackwell *et al.*, 1990 and our unpublished data) and have not been isolated by our various expression screening approaches.

Interestingly, SEF-2 has been isolated previously from a T-cell line by a very similar expression screening approach, based on using the E-box present in the LTR of the murine leukaemia virus SL3-3 (Corneliussen *et al.*, 1991). Furthermore, SEF-2 was found to be identical with the immunoglobulin transcription factor-2 identified in B-cells, which is responsible for targeting the E-box element within immunoglobulin enhancer motifs (Henthorn *et al.*, 1990). These E-boxes, like the SSTR2*inr* E-box, have a conserved guanine as the fourth nucleotide underlining again that this particular E-box interacts specifically with SEF-2 but not with other bHLH factors. Based on those early results, SEF-2 was believed to be a transcription factor having specific functions in the regulation of gene expression in the immune system. The results presented in this manuscript define a novel function for SEF-2 as a key regulator of SSTR-2 expression in neural cells. Accordingly, SEF-2 is strongly expressed in the developing and adult brain.

In conclusion, our data identify the SSTR2*inr* as a novel type of initiator element that might contact the basal transcription machinery by recruiting TFIIB through physical interaction with the bHLH transcription factor SEF-2.

Materials and methods

Cell culture and transfection

LAN-1, NGP, MCF-7, HeLa and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM). Neuro2A (N2A) cells were cultured in Earl's modified Eagle's medium (EMEM). Both media were supplemented with 10% fetal calf serum. Transient transfection assays were carried out using the standard calcium phosphate co-precipitation technique (Gorman *et al.*, 1982) as described in Pfitzner *et al.* (1995). Luciferase activity was assayed as recommended by the manufacturer (Promega) in a Luminometer ML 3000 (Dynatech). Relative light units were normalized to β-galactosidase activity (Umesono *et al.*, 1991) and protein concentration using the Bradford dye assay (Bio-Rad). Equal results were obtained with both methods. All experiments were repeated at least five times. Standard deviations were <10%.

Reporter plasmids

The hSSTR2 luciferase reporter plasmid –1800LuC was generated by inserting a 1.8 kb hSSTR2 promoter fragment at the *Bgl*II restriction site of pGL2LuC (Promega). The reporter –840LuC was constructed by inserting an 840 bp promoter fragment at the *Sac*I–*Bgl*II restriction site of pGL2LuC. The constructs –201LuC, –89LuC and –30LuC were generated by PCR amplification and inserted at the *Bgl*II restriction site of pGL2LuC. The Inr–LuC and the Inr–CAT reporter series were generated by cloning single copies of the listed oligonucleotides upstream of the pGL2LuC or the pBLCAT3 reporter genes. The same oligonucleotides were used for both the generation of the reporter constructs and as probes for the gel retardation assays:

Inr: AATCTTCCTCTTTTCCTTC**CAGATG**TCACACTGGATCC; M0: AATCTTCCTCTTTTCCTTC**CAGATG**TCACACTGG; M1: AATCTTCCTCTTTTCCTTC; M2: **CAGATG**TCACACTGGATCC; M3: AATCTTGGTCTTTTGGTTC**CAGATG**TCACACTGGATCC; M4: AATCTTCCTCTTTTGGTTC**CAGATG**TCACACTGGATCC; M5: **CAGATG**; M6: AATCTTCCTCTTTTCCTTC**CAAATG**TCACACTGGATCC; M7: TCCTTC**CAGATG**TCACACTGGATCC; M8: TCTTTTCCTTC**CAGATG**TCACACTGGATCC; M9: TCTTTTCCTTC**CAGATG**TATCCCTGGATCC; M10: AATCTTCCTCTTTTCCTTC**CATCTG**TCACACTGGATCC; M11: AATCTTCCTCTTTTCCTTC**CAGATG**TATCCCTGGATCC; M12: TCTTTTGGTTC**CAGATG**TCACACTGGATCC.

Expression vectors

CMX.PL1 and CMX.PL2 were described previously (Umesono *et al.*, 1991). To construct the CMX-SEF-2 expression plasmid, a 2.1 kb cDNA fragment encoding the full-length SEF-2 protein was inserted at the *Eco*RI–*Hin*dIII site of CMX.PL1. For construction of the mutant CMX-ΔNSEF, a cDNA fragment coding for the amino acids 369–667 was generated by PCR amplification and inserted downstream of an optimal Kozak sequence at the *Eco*RI restriction site of CMX.PL2. The expression plasmid GST-SEF-2 was obtained by PCR amplification of a cDNA encoding full-length SEF-2. The PCR product was cloned in-frame into pGEX2T (Pharmacia) as a *Bam*HI–*Eco*RI fragment. To construct the mutant GST-ΔNSEF, the respective cDNA fragment coding for the amino acids 369–667 was generated by PCR amplification and inserted at the *Eco*RI restriction site of pGEX3a (Smith and Jones, 1988). The expression plasmids GST-TBP, GST-TFIIA and GST-TFIIB were a gift from D.Reinberg. All constructs were verified by sequencing using USB Sequenase.

DNA binding studies

Preparation of whole cell extracts has been described in Buettner *et al.* (1993). The GST-fusion proteins were expressed according to Perlman *et al.* (1993). The purified GST fusion proteins (0.5 μg) or 4 μl of nuclear extract were mixed with 10 mM HEPES (pH 7.8), 80 mM KCl, 10% glycerol, 5 mM $MgCl_2$, 1 mM DTT, 0.5 μg poly(dG–dC) and incubated for 15 min on ice. Subsequently 1 ng of ^{32}P-labelled probe was added. To determine the composition of the various DNA–protein complexes, the following antibodies were included in the reaction mix: α-SEF-2 (E2-2, Dianova) and α-Flag (Kodak). After 30 min incubation on ice, the samples were run on a 4% polyacrylamide gel in 0.5× TBE at 4°C. After electrophoresis, gels were dried and subjected to autoradiography at –70°C.

Screening of genomic and λgt 11 expression libraries

A phage harbouring 20 kb of genomic sequence including the entire human *SSTR2* locus was isolated by screening a commercially available genomic library from human placental DNA (Stratagene) with a SSTR2 cDNA probe. A 2 kb *Sal*I insert reconfirmed as positive by Southern blot hybridization was cloned into pBluescript (Stratagene) and sequenced on both strands. 2×10^5 phage plaques of a commercially available mouse brain cDNA library in the vector λgt 11 (Clontech, Palo Alto,

CA) were screened using a tetramerized ^{32}P-labelled SSTR2*inr* binding site. Plating of phages and induction of lacZ fusion proteins were performed using standard protocols (Sambrook *et al.*, 1989) Briefly, upon infection, culture plates were grown for 3.5 h at 42°C and then overlayed for 5.5 h with nitrocellulose filters soaked in 10 mM IPTG. Next, duplicate filters were prepared and overlayed for another 2 h. After removal from the plates the filters were air dried and subjected to a denaturation–renaturation cycle with 6 M to 0.19 M guanidine hydrochloride in binding buffer (50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 20 mM HEPES pH 7.8). After washing in binding buffer the filters were blocked for 30 min in 5% non-fat dried milk and the binding reaction was performed for 12 h in the binding buffer supplemented with 0.25% non-fat dried milk, 5 µg/ml salmon sperm DNA, 2 µg/ml poly(dI–dC) and 1.2×10^6 c.p.m./ml of radiolabelled probe. Filters were washed three times in the binding buffer/0.25% non-fat dried milk for 5 min, dried and autoradiographed overnight. Double-positive signals were plaque purified. The specificity of binding reaction was confirmed by competition with a 50-fold molar excess of an unrelated Sp-1 oligonucleotide or the homologous SSTR2*inr* binding site.

Northern blots

To hybridize murine and human multiple tissue Northern blots (Clontech) the SEF-2 cDNA fragment spanning the nucleic acid residues 1–1795 was used as a probe. The probe does not contain the bHLH domain and therefore reacts specifically with SEF-2 mRNA. Poly(A)$^+$ RNA was prepared from Neuro2A and NGP cells and subjected to Northern blot analysis using standard procedures (Sambrook *et al.*, 1989). Hybridizations were performed in a buffer containing 50% formamide at 42°C for 16 h. The final washes were done in 0.1× SSC at 60°C for 45 min.

Primer extension assay

The reporter plasmids Inr-LuC and M6-LuC together with the empty pGL2LuC control were transfected into Neuro2A cells and total cellular RNA was prepared 12 h later. Thirty micrograms of RNA were co-precipitated with 2 pmol of ^{32}P-labelled primer, dissolved in 30 µl 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and denaturated at 90°C for 5 min. The RNA was annealed with the oligonucleotide 5′-CTTTATGTTTTTGGCGTCTTCCAT-3′ at 65°C for 90 min. The annealed primer was extended using 5 U reverse transcriptase (AMV, Boehringer) in 50 µl reactions containing 50 mM Tris–HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP and 10 U human placental RNase inhibitor (Boehringer) at 42°C for 90 min. The reaction was stopped by adding 1 µl 0.5 M EDTA and 10 µl RNase A . The extended products were ethanol precipitated, washed with 70% ethanol and analyzed on a 6% denaturing polyacrylamide gel in 0.5× TBE at RT.

RNase protection assay

The nuclease protection assays were performed as described previously (Bruno *et al.*, 1992, 1994). A *Bgl*II–*Pst*I fragment of the *hSSTR2* gene was subcloned in pWW152, a vector related to pBluescript KS+ (Stratagene). The construct was linearized with *Hin*dIII and antisense RNA was generated using T7 RNA polymerase. The resulting riboprobe has a length of 357 nucleotides. Solution hybridization/nuclease protection assay was performed using 50 µg total cellular RNA from LAN-1, NIH 3T3 and MCF-7 cells or 50 µg yeast tRNA.

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