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The DNA sequence encompassing the transcription start site of a TATA-less promoter contains enough information to drive neuron-specific transcription

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ABSTRACT

The FE65 gene encodes a nuclear protein of unknown function that is expressed in several areas of the rat nervous system during development and in the adult animal, particularly in somatic and visceral ganglia. FE65 mRNA is abundant in neuronal cell lines, whereas it is barely detectable in non-neuronal cells. We identified the two transcription start sites of theFE65 gene and we isolated the rat genomic fragment containing one of these two transcriptional start sites. We demonstrate that this fragment contains a promoter able to direct an efficient transcription of a reporter gene in PC12 cells and in NTERA2 cells upon their differentiation with retinoic acid, whereas it functions poorly in non-neuronal cells, such as Rat2 fibroblasts and BRL hepatocytes. This promoter is composed of two regions. The first includes a *cis*-element whose removal greatly decreases the transcriptional efficiency in all cells examined and which forms similar complexes with proteins from PC12 and Rat2 cells. This *cis*-element binds Sp1 or another GC-binding factor. The second *cis*-element encompasses the transcription start site and is still able to direct transcription only in neuronal cells. The DNA–protein complexes formed by this *cis*-element in neuronal cells differ from those formed in non-neuronal cells. The analysis of point mutations in this region indicates that the proteins that bind to this *cis*-element interact with both overlapping and distinct nucleotide sequences.

INTRODUCTION

The mechanisms underlying brain-specific transcription are still obscure compared to those responsible for specific gene expression in other tissues. This is in part due to the complexity of the nervous system, which is a mosaic of various types of cells that express different sets of genes, covering most of the expressed mammalian genome (1). Most of the data on brain-specific transcription concerns the characterization of transcriptional factors restricted to the central nervous system or to its regions, identified on the basis of their similarity to other factors regulating the developmental gene programs in invertebrate organisms (for a review see 2), and only a few results are available on the dissection of brain-specific promoters and on the transcriptional factors involved in their regulation (reviewed in 2, 3).

We are studying the brain-specific expression of several cDNAs isolated by differential screening from a rat brain cDNA library (4). One of these cDNAs, designated FE65, hybridizes to an mRNA almost exclusively present in the central nervous system. *In situ* hybridization experiments in mouse embryos showed that the FE65 gene is expressed in specific regions of the nervous system, particularly in the spinal and visceral ganglia, and in gangliar structures of the encephalon and of sense organs (5). This expression is very precocious, being already evident in the embryonic precursors of the gangliar structures.

RNase protection experiments revealed two FE65 mRNA species that differ by only six nucleotides, as a consequence of an alternative splicing: the longest FE65 mRNA is abundant and neuron-specific (present in PC12 cells and in rat brain), whereas the shortest one is scarce and ubiquitous (6). A polyclonal antibody directed against the recombinant polypeptide encoded by the FE65 cDNA recognizes a nuclear protein in PC12 cells, and the open reading frame of this mRNA shows an amino acid sequence that has a long stretch of homology with the DNA binding domain of a retroviral protein (6). The amino acid region flanking this homology is able to activate transcription when fused to a heterologous DNA binding domain (6).

Herein we report the isolation of the FE65 gene and demonstrate that one of the two FE65 gene promoters drives a very efficient transcription in neuronal cell lines, but not in non-

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neuronal cells, and that factors interacting with, or near to, the initiator sequence are probably involved in the cell-specific differences in promoter efficiency.

MATERIALS AND METHODS

cDNA and gene isolation and sequencing

Double-stranded cDNA was prepared from rat brain poly(A^+) using a cDNA synthesis kit (Amersham) according to the supplier's instructions with random oligonucleotides as primers (7). An asymmetric polymerase chain reaction (PCR) was performed to synthesize the minus strand of the FE65 cDNA (6) using an oligonucleotide complementary to the FE65 mRNA (oligo REV: 5′-CATGCCAAATAGGAGGCTAGCAC-3′; see Fig. 1). The PCR protocol was 1 min at 92°C, 30 s at 52°C and 2 min at 72°C for 20 cycles. After the Taq polymerase was removed, the DNA molecules present in the mixture were tailed at the 3′ extremities using a deoxyterminal transferase kit (Boehringer) according to the supplier's instructions, in the presence of 0.1 mM dGTP. Similarly 0.2 pmol of an unrelated oligonucleotide (oligo FORW: 5′-GCAAGCTTGGGACAGG-ATGARTGACCTTGGT-3′) were tailed at the 3′ extremity with 0.1 mM dCTP and deoxyterminal transferase. The G-tailed cDNA mixture was the template for synthesis of the plus strand cDNA using the C-tailed oligo FORW as a primer. 0.2 pmol of C-tailed oligo FORW plus 2 μg of asymmetrically amplified and G-tailed cDNA were incubated with 1×PCR buffer (Perkin-Elmer) at 95°C for 10 min, then slowly cooled to 60°C. The plus strand polymerization was performed at 72°C for 30 min with 0.2 mM of each dNTP and 2.5 units of Taq polymerase (Perkin-Elmer). After the removal of excess oligonucleotides by gel filtration chromatography and of Taq polymerase by proteinase K treatment, the double-stranded cDNA was amplified using the oligo REV and the untailed oligo FORW (0.2 pmol each) with the following PCR protocol: 1 min at 95°C, 1 min at 60°C, 2 min at 72°C for 30 cycles. The amplified DNA was then ligated into pGEM3Z vector (Promega). The resulting recombinant clones were screened by hybridizing them to the labelled FE65 cDNA (6). The plasmids present in four positive clones were sequenced by the dideoxynucleotide method (8) on both strands.

A rat liver genomic library in λEMBL3A was screened according to standard procedures (7) with the 5′ cDNA end, cloned as described above, as a primer. One genomic clone was found and characterized by partial nucleotide sequencing. A region of 2 kb 5′ flanking the transcription start site was sequenced on both strands.

Primer extension and RNase protection experiments

A standard protocol was used for the primer extension analysis (7). The oligonucleotide used for the primer extension was complementary to the sequence double underlined in Fig. 1, and labelled by T4 polynucleotide kinase and [γ-^{32}P]ATP (Amersham, sp. act. 3000 Ci/mmol). RNase protection experiments were performed as described (6). The probe was an antisense RNA labelled with [α-^{32}P]ATP (Amersham, sp. act. 400 Ci/mmol), synthesized *in vitro* using T7 RNA polymerase (Promega) and, as template, the first 130 bp of the cDNA of Fig. 1.

Plasmids, cells and transfections

The plasmids for the transfection experiments were constructed by cloning into the pCAT Basic vector (Promega) different fragments of DNA from the isolated genomic clone, either by using compatible restriction sites or by amplifying the specific DNA region by PCR. The oligonucleotides thus used were designed according to the sequence of the genomic DNA, and all the inserts cloned into the pCAT Basic vector and derived

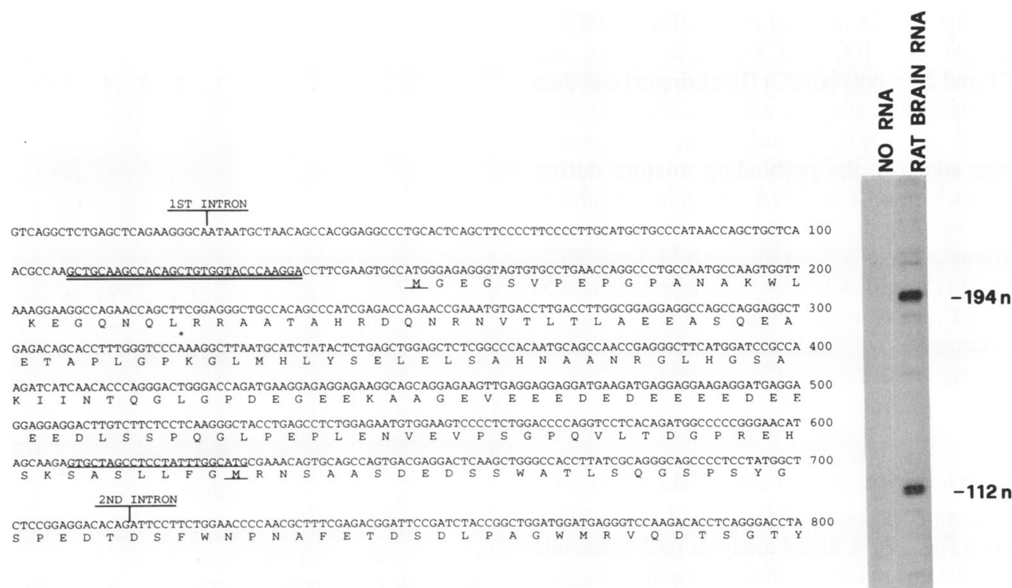

Figure 1. Nucleotide sequence of the 5′ end of the FE65 cDNA and primer extension analysis. The underlined sequence is complementary to the oligonucleotide (oligo REV, see Materials and Methods) used in the PCR experiments performed to isolate the full-length cDNA. The double underlined sequence is complementary to the oligonucleotide used in the primer extension analysis. The positions of two introns in the corresponding genomic sequence are indicated.

from a PCR amplification were sequenced on both strands. In order to exclude the generation of *cis*-elements at the cloning site in the plasmid containing the DNA sequence from −55 to +44 (see Results), a 125 bp *Hind*III fragment from λ genomic DNA was inserted upstream from nucleotide −55.

Cells were grown in a 5% CO_2 atmosphere at 37°C. The culture media were as follows. PC12: DMEM supplemented with 10% fetal calf serum (Hyclone) and 5% horse serum (Gibco) and 1% antibiotics. RAT2 and BRL: DMEM supplemented with 5% calf serum (Gibco). NTERA2: DMEM supplemented with 10% fetal calf serum. NTERA2 cells were treated with retinoic acid as described in (9).

Transfections were performed with the calcium phosphate method as previously described (6). CAT assays were performed as described (6) and the enzyme activity was measured as the amount of chloramphenicol converted into its acetylated forms and reported as the percentage of the CAT activity measured when the pSV2CAT plasmid (SV40 early promoter controlling transcription of the *CAT* gene) was transfected in the different cell lines. Two different preparations of each plasmid were used.

Electrophoretic mobility-shift assays

Whole cell extracts used for mobility-shift assays were prepared according to (10). Briefly, cell plates were washed twice with phosphate buffer solution, the cells were scraped from the plates and lysed by the addition of 100 μl/100 mm plate of a buffer containing 20 mM Tris−HCl, pH 7.5, 20% glycerol, 2 mM DTT and 400 mM KCl. Two freezing and thawing cycles were performed and, after 10 min centrifugation at 4°C, the supernatants were stored in aliquots at −80°C.

The different DNA fragments used as probes in the gel shift experiments were prepared by labelling double-stranded DNA (1 pmol) with 16 U of T4 polynucleotide kinase (Promega) and 40 μCi of [γ-^{32}P]ATP (Amersham, sp. act. 3,000 Ci/mM) at 37°C for 30 min. Labelled fragments (20,000−40,000 c.p.m., corresponding to 1 fmol) were used for each reaction. Binding reactions were performed by incubating on ice 5 μg of protein from cellular extracts for 15 min in 18 μl of a buffer containing 10 mM Tris−HCl, pH 7.5, 100 mM KCl, 2% Ficoll, 6% glycerol, 1 mM DTT and 2 μg poly(dI/dC) (Boehringer) and then for 15 min at room temperature after the addition of the labelled oligonucleotide in 2 μl. Where indicated, competitor oligonucleotides were added to the prebinding mixture during the first 15 min of incubation. The DNA−protein complexes were separated from the unbound DNA probe on a 4% non-denaturing polyacrylamide gel in 0.5× TBE (44 mM Tris−HCl, 44 mM boric acid, 12.5 mM EDTA). Electrophoresis (20 mA/150 V) was carried out at room temperature.

The nucleotide sequences of Sp1 and CTF/NF1 oligonucleotides are those reported in (11, 12), respectively.

RESULTS

Determination of the transcription start sites of the FE65 gene

Fig. 1 shows the nucleotide sequence of the 5′ end of the FE65 cDNA, identified via a RT−PCR based analysis (see Materials and Methods), that allowed us to correct the previously published sequence (6). Primer extension analysis, performed using as a primer an oligonucleotide complementary to the sequence double underlined in Fig. 1, revealed two different 5′ ends, one located 112 nucleotides upstream from the primer and included in the cloned cDNA, and another located 194 nucleotides upstream, in a region not included in the sequenced cDNA (see Fig. 1). RNase protection experiments confirmed this finding. In fact, as shown in Fig. 2, an antisense RNA probe that covers the first 130 nucleotides of the cDNA is protected by two different mRNAs, the shorter one (band b of Fig. 2) corresponding to that identified with the primer extension analysis and included in the cDNA sequence presented. The ratio between the amounts of these two mRNAs is about 1 (both in the primer extension and in the RNase protection assays) and both forms are abundant in rat brain and PC12 RNAs and scarce in rat fibroblast RNA. This confirms previously published results indicating that FE65 mRNA is not completely restricted to neural cells (6), and demonstrates that both the starting sites are used similarly.

Isolation of a rat genomic fragment containing one of the two transcription start sites

The first 390 nucleotides of the cDNA were used as a probe to screen a rat genomic library. This allowed the isolation of a genomic clone (λ65.2), partially overlapping with the previously reported genomic clone λ65.1 (6). Clone λ65.2 contains four exons: the first one covers the cDNA sequence from nucleotide 26 to nucleotide 715 (see Fig. 1) and the other three exons represent the first exons of clone λ65.1. Nucleotide 26 of the cDNA corresponds to one of the two start sites identified by the primer extension (see Fig. 1), and it represents the 5′ extremity of the mRNA that protects the RNA antisense probe of Fig. 2

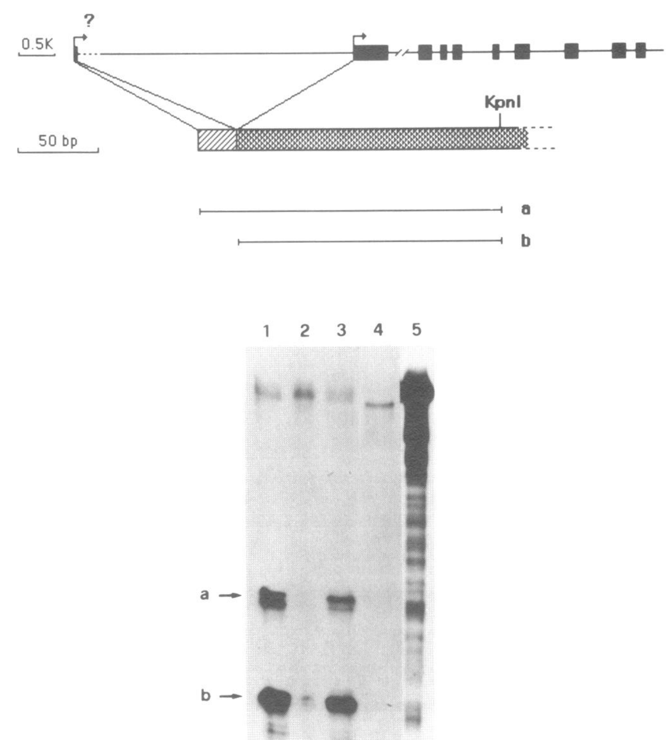

Figure 2. RNase protection experiment. In the upper part of the figure is a schematic representation of the structure of the FE65 gene together with the RNA probe. Two fragments of this probe are protected (a and b); band a is 130 nucleotides long and band b is 106 nucleotides. The RNA used in the experiments are: lane 1, PC12 cell RNA; lane 2, Rat2 cell RNA; lane 3, rat brain RNA; lane 4, yeast tRNA. In lane 5 the undigested probe (180 nucleotides, of which 130 belong to FE65 cDNA and 50 to the pGEM3Z polylinker) is run.

(giving rise to band b). The insert of clone λ65.2 contains about 3.6 kb of genomic DNA that flank this first exon, but they do not contain the first 25 bp of the cDNA reported in Fig. 1.

Cell type-specific promoter function of the isolated genomic fragment

In order to evaluate whether the sequence flanking the first exon present in the genomic clone and containing one of the two start sites is able to promote transcription, we generated several constructs in which *CAT* gene transcription is under the control of several deletion mutants containing different fragments of the genomic region flanking the transcription start site. Figure 3 shows the results of the transfection experiments performed with these plasmids, and demonstrates that a DNA region constituted of 163 bp upstream and 44 bp downstream of the transcription start site is able to drive a very efficient transcription in PC12 pheochromocytoma cells. In contrast, the transcriptional efficiency of this construct is poor in Rat2 fibroblasts and in BRL hepatocytes. There was no difference between the construct containing 2,000 bp and that containing 163 bp upstream from the transcription start site. With a *CAT* construct containing 55 bp upstream and 44 bp downstream of the start site, the transcriptional efficiency was significantly decreased in all the three cell lines examined. This demonstrates that the region between nucleotide −163 and nucleotide −55 contains a DNA element important for promoter activity. However, these 55 bp upstream (and similarly only 13 bp upstream; see below) plus 44 bp downstream of the start site are still able to drive a transcription significantly stronger in PC12 than in Rat2 or in BRL cells. In order to exclude the artifactual generation of a *cis*-element at the cloning site, a 125 bp *Hin*dIII fragment of the λ genome was inserted upstream of the −55 to +44 fragment. The resulting *CAT* construct was transcribed with the same efficiency as the above described −55 to +44 *CAT* clone (data not shown).

The FE65 gene is highly conserved among eukaryotic organisms (6), therefore we performed similar experiments in NTERA2 human teratocarcinoma cells; most of these cells differentiate into neurons following treatment with retinoic acid (9), and we previously demonstrated that human FE65 mRNA is several-fold induced upon the appearance of this differentiated phenotype (5). Therefore, we tested if retinoic acid-induced differentiation was accompanied in these cells by increased transcription driven by the above described promoter. Fig. 4

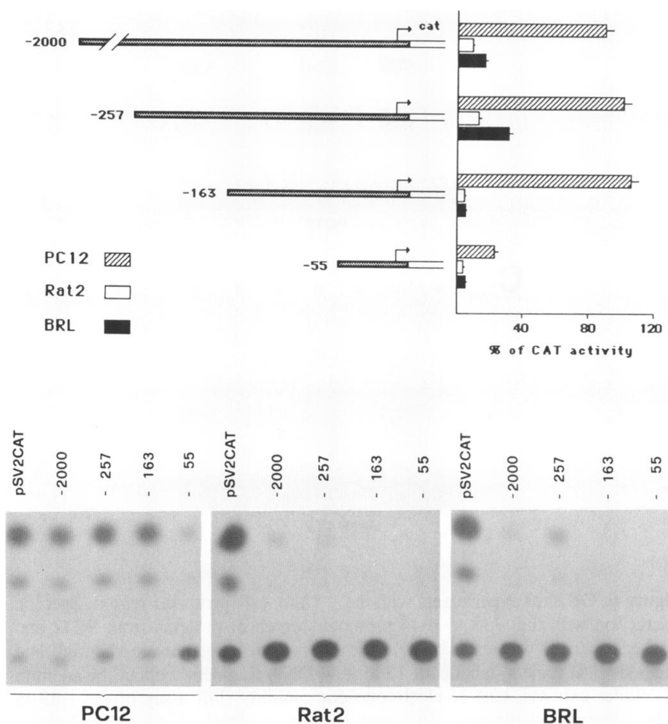

Figure 3. FE65 promoter activity in neuronal and non-neuronal cells. The upper part of the figure shows a schematic representation of the deletion mutants with their relative CAT activities. The reported CAT activities represent the mean values of four distinct transfection experiments and are reported as a percentage of the CAT activity obtained by transfecting with the pSV2CAT plasmid. The standard error of each mean value is reported on the top of the bar. The lower part of the figure shows a representative example of a CAT assay.

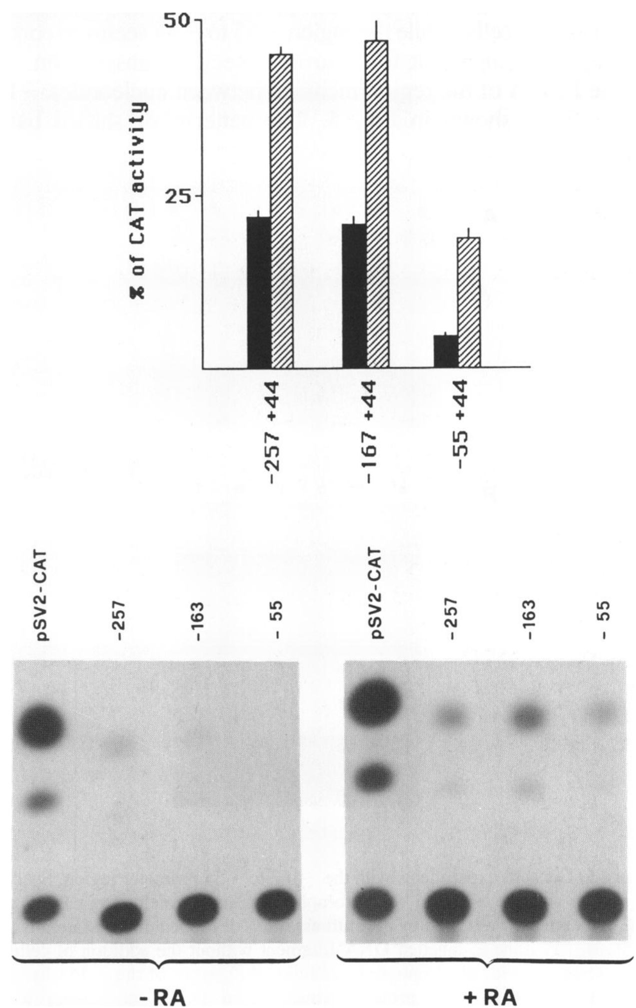

Figure 4. FE65 promoter activity in differentiated and undifferentiated NTERA2 cells. In the upper part of the figure is a diagram of the mean values, of four distinct transfection experiments, of the CAT activities obtained by transfecting NTERA2 cells with same deletion mutants used in the experiments of Fig. 3. CAT activity was calculated as described in Fig. 3 and reported as a percentage of the CAT activity obtained by transfecting with the pSV2CAT plasmid. The black bars refer to undifferentiated cells, and the hatched bars to differentiated cells. The standard error of each mean value is reported on the top of the bar. In the lower part of the figure is a representative example of a CAT assay. −RA, undifferentiated cells; +RA, differentiated cells.

shows the results of transfections of the same constructs used in the experiments reported in Fig. 3 into NTERA2 cells treated with retinoic acid for 12 days. Transcription efficiency was significantly increased in retinoic acid-treated cells compared to untreated cells, and this phenomenon was observed also with the construct containing only 55 bp upstream plus 44 bp downstream of the transcription start site. Also, these results indicate that the removal of the sequence from −163 to −55 causes a decrease in the transcriptional efficiency in both differentiated and undifferentiated cells.

Trans-acting factors that bind to FE65 promoter

Analysis of the deletion mutants demonstrates that the DNA sequences involved in regulation of the FE65 promoter are located in a genomic region spanning from nucleotide −163 to nucleotide +44. The sequences between nucleotides −163 and −55 contain *cis*-element(s) that interact with transcriptional factor(s) responsible for enhanced transcription both in neuronal and in non-neuronal cells, while the region −55 to +44 seems to contain *cis*-elements important for neuronal-specific transcription.

The EMSA of the region included between nucleotides −163 and −55 is shown in Fig. 5. The patterns of shifted bands obtained with PC12 and Rat2 extracts are similar, which is in agreement with the observation that removal of this sequence affects transcriptional efficiency in both PC12 and in Rat2 cells. Computer-assisted analysis of the promoter sequence indicated that the region from nucleotide −163 to nucleotide −55 contains one Sp1 binding site and two CTF/NF1 sites (see Fig. 7). In order to ascertain whether these transcriptional factors are involved in the formation of the observed complexes, competition experiments were performed by using two oligonucleotides containing the consensus sequences for the binding of Sp1 or CTF/NF1 (see Materials and Methods). These competition experiments showed that the oligonucleotide containing the Sp1 *cis*-element specifically competes for the formation of most of the protein−DNA complexes present in the EMSA pattern; in

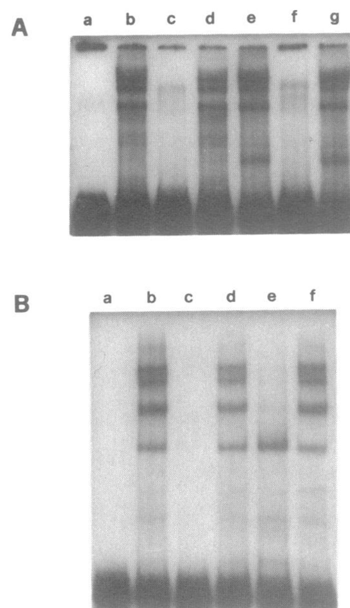

Figure 5. Gel shift experiments with the −163 to −55 promoter region. Specific interaction with the −163 to −55 promoter region of proteins from PC12 and Rat2 cell extracts was tested by gel shift assays as described under Materials and Methods. (A) Lane a, labelled DNA fragment without the addition of cellular extracts; lanes b−d, electrophoretic mobility shift assay of the −163 to −55 fragment challenged with protein extracts from PC12 cells; lanes e−g, electrophoretic mobility shift assay of the −163 to −55 fragment challenged with protein extracts from Rat2 cells; lanes c and f, binding competed by a 30-fold molar excess of the unlabelled −163 to −55 fragment; lanes d and g, binding competed by a 30-fold molar excess of an unrelated DNA fragment (125 bp long *Hin*dIII fragment of λ genomic DNA). (B) Lane a, labelled DNA fragment without the addition of cellular extracts; lanes b−f, electrophoretic mobility shift assay of the −163 to −55 fragment challenged with protein extracts from PC12 cells; lane c, binding competed by a 30-fold molar excess of the unlabelled −163 to −55 fragment; lane d, binding competed by a 30-fold molar excess of an unrelated DNA fragment (125 bp long *Hin*dIII fragment of λ genomic DNA); lane e, binding competed by a 30-fold molar excess of the Sp1 oligonucleotide; lane f, binding competed by a 30-fold molar excess of the CTF/NF1 oligonucleotide.

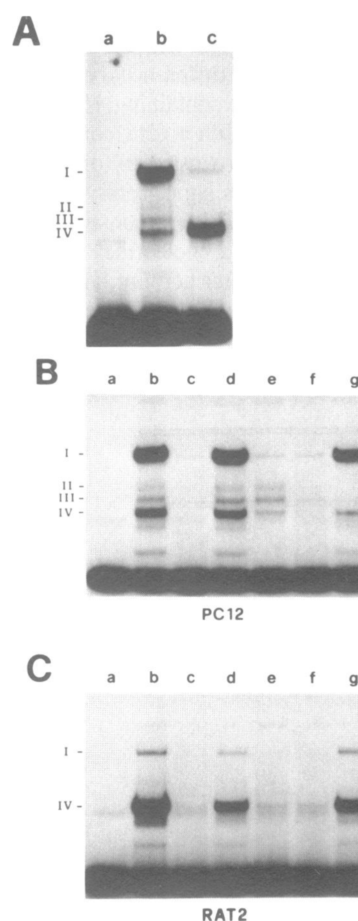

Figure 6. Gel shift experiments with the −13 to +44 promoter region. Specific interaction with the −13 to +44 promoter region of proteins from PC12 and Rat2 cell extracts was tested by gel shift assays as described under Materials and Methods. (A) Lane a, labelled −13 to +44 DNA fragment without the addition of cellular extracts; lane b, electrophoretic mobility shift assay of the −13 to +44 fragment challenged with protein extracts from PC12 cells; lane c, electrophoretic mobility shift assay of the −13 to +44 fragment challenged with protein extracts from Rat2 cells. (B) Lane a, labeled −13 to +44 DNA fragment without the addition of cellular extracts; lanes b−g, electrophoretic mobility shift assays of the −13 to +44 fragment challenged with protein extracts from PC12 cells; lane c, binding competed by the unlabelled −13 to +44 fragment; lanes d−g, binding competed by a 30-fold molar excess of the unlabelled mutant oligonucleotides MUT1, MUT2, MUT3 and MUT4, respectively (for the sequences of the competitor oligonucleotides see Fig. 7). (C) As (A) but using extracts from Rat2 cells.

contrast, the CTF/NF1 oligonucleotide does not compete (see Fig. 5, panel B).

The interaction of transcriptional factors with the region that includes the transcription start site (from −55 to +44) was examined by gel shift experiments in which protein extracts from PC12 and Rat2 were tested. With PC12 extract there were at least four specifically shifted bands (bands I, II, III and IV in panel A of Fig. 6), whereas with the Rat2 extract there were only two major shifted bands, whose electrophoretic migrations correspond to those of bands I and IV obtained with the PC12 extract. Furthermore, the relative abundance of these two bands is different in PC12 compared to Rat2 extracts: band I predominates with PC12 extracts, whereas band IV is more abundant than band I with the extract from Rat2 cells. These gel shift experiments were also performed with two other probes (from −27 to +44 and from −13 to +44) and the patterns of the bands shifted were identical with all of these three probes. Gel shift experiments performed with extracts from BRL cells give results similar to those obtained with Rat2 extracts (data not shown).

To identify the nucleotides involved in the DNA−protein interaction in this region, competition experiments have been performed with four mutant oligonucleotides (see Fig. 7). With PC12 cell extracts, the MUT1 oligonucleotide, in which the start site (A_{+1}, T_{+2},T_{+3}) is changed, does not compete for any of the four bands (see Fig. 6, panel B, lanes d). The MUT2 oligonucleotide, bearing a mutation of nucleotides A_{+4}, A_{+5} and T_{+6} efficiently competes for band I, does not compete at all for bands II and III, and competes poorly for band IV (see Fig. 6, panel B, lanes e). The MUT3 oligonucleotide competes for band I but competes poorly for bands II and III (see Fig. 6, panel B, lanes f). Lastly, the MUT4 oligonucleotide, in which G_{+28}, A_{+30} and T_{+32} are mutated, does not compete for bands I and IV but competes totally for bands II and III (see Fig. 6, panel B, lanes g). The results of competition experiments performed with Rat2 cell extracts were compatible with those obtained using PC12 cell extracts: oligonucleotides MUT1 and MUT4 do not compete for the formation of complexes I and IV, whereas oligonucleotides MUT2 and MUT3 do (Fig. 6, Panel C).

The mutant oligonucleotides used in the gel shift experiments were cloned into the pCAT Basic vector in order to test their ability to drive *CAT* gene transcription (Fig. 8). The wt −13 to +44 region was able to drive *CAT* gene transcription in PC12 cells, whereas the MUT1 oligonucleotide cloned upstream of the *CAT* gene was not. In contrast, in PC12 cells the mutations present in the MUT2 and MUT4 oligonucleotides caused only a moderate decrease in the transcriptional efficiency of the −13 to +44 promoter.

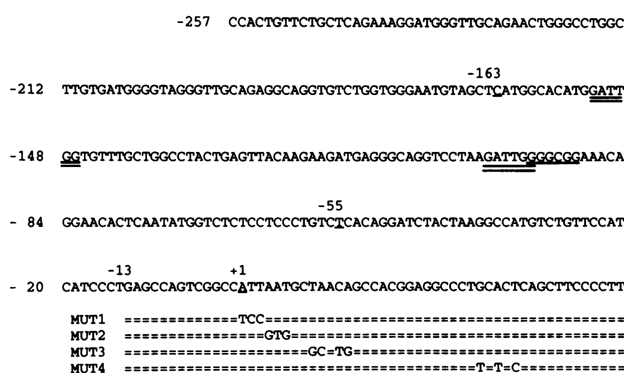

Figure 7. Nucleotide sequence of the FE65 promoter from nucleotide −257 to nucleotide +44. A corresponds to the start site. The mutations present in the oligonucleotides used in gel shift competition experiments are reported. The Sp1 *cis*-element is underlined; CTF/NF1 *cis*-elements are double underlined.

DISCUSSION

The results reported in this paper demonstrate that the FE65 gene promoter is able to direct the transcription of a reporter gene in a cell-specific fashion, so that in PC12 and in differentiated NTERA2 cells the promoter efficiency is high, whereas in RAT2 fibroblasts, in BRL hepatocytes and in undifferentiated NTERA2 cells it functions at a very low rate. The dissection of the FE65 gene promoter allowed us to identify two functional regions: the first spans from nucleotide −163 to nucleotide −55 and contains *cis*-elements whose removal causes a significant decrease in transcriptional efficiency in PC12 and in RAT2 cells; the second functional region contains *cis*-elements that are restricted to a small DNA region encompassing the transcription start site from nucleotide −13 to nucleotide +44 and that are able to drive efficient transcription only in neuronal cells.

The FE65 promoter described belongs to the family of TATA-less promoters, which are mainly promoters of housekeeping genes. However, some tissue-specific genes lack the TATA consensus, e.g. the synapsin I gene (13), the brain-specific aldolase C gene (14), the nerve growth factor receptor gene (15), the leukocyte integrin alpha subunit (16) and the lymphocyte CD4 gene (17). It is well documented that initiator elements (Inr), which encompass the transcription start site, are able to direct transcription in the absence of binding sites for other transcription factors in either TATA-containing or TATA-less promoters (for a review see 18). The sequence homology between the FE65 start

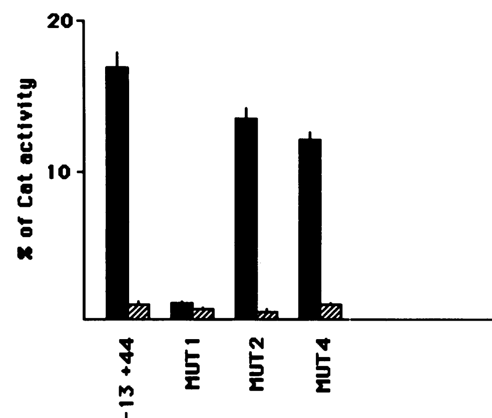

Figure 8. Effect of mutations on transcriptional efficiency of the minimal FE65 gene promoter. The diagram shows the mean values (from three different experiments) of CAT activity, calculated as described in Fig. 3 as a percentage of the CAT activity obtained transfecting with the pSV2CAT plasmid. −13 +44 refers to the construct containing the minimal promoter of the FE65 gene cloned into the pCAT Basic vector, MUT constructs contain the oligonucleotides reported in Fig. 7 cloned into pCAT Basic. The black bars refer to PC12 cells and the hatched bars to Rat2 cells. The standard error of each mean value is reported on the top of the bar.

site and several Inr sequences described so far includes the nucleotides $_{-2}CC\underline{A}TT_{+3}$, that fits with the consensus sequence for Inr YCANTNT (18). The interesting characteristic of the FE65 promoter is the ability of the small region that encompasses the transcription start site to drive a significant tissue-specific basal transcription. This region is probably responsible for the tissue specificity of the entire promoter, as has been observed in a few other cases (17, 19). Similarly, there are a few examples of genes whose regulation during development involves the Inr sequence (20–23).

The nucleotide sequence of the region between nucleotides −163 and −55 contains a GC-rich sequence representing the consensus element for the binding of Sp1. Competion experiments demonstrated that Sp1 (or another GC binding protein) is involved in the formation of DNA–protein complexes with the −163 to −55 region. This is in agreement with the results of the transfections indicating that the −163 to −55 fragment contains promoter element(s) which enhances transcriptional efficiency, regardless of the cell used in the experiment.

The electrophoretic mobility shift assay of the −55 to +44 region revealed tissue-specific differences in the DNA–protein complexes between PC12 and Rat2 cells. The differences are restricted to *cis*-elements located in the −13 to +44 region and they are of two types: (i) a qualitative difference represented by two additional bands that are shifted with PC12 extracts with respect to Rat2 extracts (bands II and III of Fig. 6); (ii) a quantitative difference in the two bands common to PC12 and Rat2 extracts (bands I and IV of Fig. 6).

The analysis of point mutations of the −13 to +44 region suggests that more than one factor interacts with this region, giving rise to different DNA–protein complexes, and that these factors interact both with overlapping and with distinct nucleotide sequences. In fact, nucleotides A_{+1}, T_{+2} and T_{+3} do not seem to be indispensable for any of the protein–DNA complexes formed. The nucleotides from +4 to +13 are needed for the formation of the complexes of bands II and III, and the nucleotides from +28 to +32 are necessary for the binding of the proteins present in the complexes of bands I and IV, but their mutation does not affect the formation of the complexes of bands II and III. This suggests that, if nucleotides A_{+1}, T_{+2} and T_{+3} are involved together with the nucleotides from +4 to +13 in the formation of the complexes of bands II and III, they are not involved at the same time in the formation of the complexes I and IV. Similarly, if nucleotides A_{+1}, T_{+2} and T_{+3} are involved in the formation of complexes I and IV they cannot be available for the binding of the protein(s) responsible for the shift of bands II and III.

Transfections in PC12 cells of the MUT2-CAT construct, in which the oligonucleotide MUT2 does not form complexes II and III, and of the MUT4-CAT construct, in which the mutations prevent the formation of complexes I and IV, result in only a slight decrease in transcriptional efficiency compared to the wild-type FE65 minimal promoter. This suggests that not only complexes II and III, which are PC12-specific, have a positive effect on transcription, but also that complexes I and IV are sufficient for the activation of transcription in PC12 cells. Furthermore, the transcriptional efficiency of a chimeric construct containing the SV40 enhancer upstream from this −13 to +44 region indicates that this fragment contains both positive and negative elements. In fact, in PC12 cells the efficiency of this construct was higher than that of the wild-type SV40 early promoter, whereas in Rat2 fibroblasts its transcriptional efficiency was significantly lower than that of pSV2CAT, which contains the SV40 enhancer in its wild-type context (data not shown). Thus, it seems conceivable that the ratio between complex I and complex IV plays a key role in the regulation of promoter efficiency: the prevalence of complex IV is associated with promoter inhibition, whereas the prevalence of complex I is associated with promoter activation. This is compatible with the observation that Inr binding proteins can either activate or repress transcription (24).

We were unable to detect point mutations that prevented the formation of complex I but not of complex IV or of complex IV but not of complex I. This means that either two different factors bind to the same sequence or the same factor undergoes post-translational modification, thereby causing the different electrophoretic behaviour and the different activity.

The proteins interacting with the Inr sequence of the FE65 gene have not beeen identified. Several Inr binding proteins have been reported: TFII-I binds to the Inr of adenovirus major late promoter (25) and also interacts with the TATA binding protein in the absence of a TATA box (26); HIP-1 (E2F) binds to the Inr sequence of the murine gene encoding dihydrofolate reductase (27) and to the adenovirus E2 promoter (28); YY1 interacts with the Inr of the adeno-associated virus P5 and is physically associated with the transcriptional factor Sp1 (29). However, evidence that these transcriptional factors have any role in tissue-specific gene regulation is lacking. The results reported in this paper suggest that neuron-specific factors, interacting with FE65 Inr, could be responsible for the transcriptional regulation of this gene. Considering the early appearance of FE65 transcripts during embryonic development and its precocious compartimentalization, it will be of interest to test the involvement of the factors interacting with FE65 Inr in the turning on of this gene.

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