DNA-binding protein Pur α and transcription factor YY1 function as transcription activators of the neuron-specific FE65 gene promoter

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Fe65 is an adaptor protein that interacts with the Alzheimer β -amyloid precursor protein and is expressed mainly in the neurons of several regions of the nervous system. The FE65 gene has a TATA-less promoter that drives an efficient transcription in cells showing a neuronal phenotype, whereas its efficiency is poor in non-neuronal cells. A short sequence encompassing the transcription start site contains sufficient information to drive the transcription in neuronal cells but not in non-neuronal cells. Electrophoretic mobility-shift assays performed with rat brain nuclear extracts showed that three major DNA–protein complexes, named BI, BII and BIII, are formed by the FE65 minimal promoter. The proteins present in complexes BI and BII were purified from bovine brain; internal microsequencing of the purified proteins demonstrated that they corresponded to the

previously isolated single-stranded-DNA-binding protein Pur α , abundantly expressed in the brain. In Chinese hamster ovary (CHO) cells, where the efficiency of FE65 promoter is very low, transient expression of Pur α increased the transcription efficiency of the FE65 minimal promoter. By using oligonucleotide competition and a specific antibody we demonstrated that the transcription factor YY1 is responsible for the formation of complex BIII. Also in this case, the transient expression of the YY1 cDNA in CHO cells resulted in an increased transcription from the FE65 minimal promoter. The absence of any co-operative effect when CHO cells were co-transfected with both YY1 and Pur α cDNA species suggests that two different transcription regulatory mechanisms could have a role in the regulation of the FE65 gene.

INTRODUCTION

FE65 cDNA was first isolated by using as a probe an expressed sequence tag that belongs to a collection of expressed sequence tags isolated by differential screening of a rat brain cDNA library [1,2] and encodes a 90 kDa protein with the characteristics of an adaptor molecule: in fact, it possesses three protein–protein interaction domains, two PID/PTB domains [3] and a WW domain [4] respectively. Through one of the two PID/PTB domains Fe65 protein interacts with the intracellular region of the Alzheimer β -amyloid precursor protein [5,6], whereas the other two protein–protein interaction domains bind to polypeptides, which are currently under examination. The relevance of this protein network in the generation of the Alzheimer's disease

phenotype is suggested by the finding that Fe65 interacts poorly with the mutant forms of β -amyloid precursor protein responsible for some cases of familial Alzheimer's disease [6].

The FE65 gene shows a privileged expression in the neurons of several regions of the mouse nervous system, mainly in ganglionic structures of somatic, visceral and sense organ systems, and in the brain, particularly in the cortex and in the hippocampus [7]. During mouse development the onset of expression of this gene takes place around embryonic day 10 and is restricted to specific embryonic structures [7]. Low levels of FE65 mRNA are also present in some non-neuronal tissues, such as heart and lung (R. Faraonio and T. Russo, unpublished work). Regulation of transcription is a key element in the generation of the differentiated phenotype in both neural and non-neuronal tissues;

therefore the identification of *cis*- and *trans*-acting factors involved in the transcription regulation might contribute to an understanding of the molecular mechanisms underlying cellular differentiation. The TATA-less promoter of the FE65 gene drives the transcription of a reporter gene in cultured cells of neural origin, whereas its efficiency in non-neuronal cells is poor. We defined the minimal promoter region which is still able to function in neural cells; this corresponds to a short region (from nt –13 to nt +44) encompassing its transcription start site [8]. The analysis of mutant FE65 gene promoters suggests that a few nucleotides surrounding the transcription start site are crucial for the activity of the promoter [8].

When incubated with nuclear extracts from rat brain or from cultured cells of neural origin, the –13 to +44 region forms several DNA–protein complexes; here we describe the identification of two proteins responsible for the formation of these complexes. They are the transcription factors Pur α and YY1. The overexpression of either Pur α or YY1 cDNA species in non-neuronal cells results in the activation of the transcription driven by FE65 minimal promoter.

MATERIALS AND METHODS

Preparation of extracts

Nuclei either from rat or bovine brains were prepared by a published procedure [9], with some modifications. All operations were performed at 4 °C; brain tissues were cut into small pieces, washed in saline solution and homogenized in 5 ml/g of tissue in

Abbreviations used: CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; CMV, cytomegalovirus; d.s., double-stranded; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; pRb, retinoblastoma protein; s.s., single-stranded; TFA, trifluoroacetic acid.

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buffer I [15 mM Hepes (pH 7.9)/10 mM KCl/5 mM MgCl_2 /0.1 mM EDTA/0.5 mM EGTA/350 mM sucrose]. The homogenate was filtered through sterile gauze and centrifuged to pellet nuclei. Nuclei were washed with buffer I, centrifuged again and resuspended in buffer C [20 mM Hepes (pH 7.9)/25% (v/v) glycerol/0.42 M NaCl/1.5 mM MgCl_2 /0.2 mM EDTA/0.5 mM dithiothreitol (DTT)/0.5 mM PMSF] for nuclear extract preparation performed as described in [10]. Finally, the nuclear extracts were dialysed against dialysis buffer [50 mM Hepes (pH 7.9)/12.5 mM MgCl_2 /1 mM EDTA/17% (v/v) glycerol/100 mM KCl/1 mM DTT].

Whole cell extracts were prepared as described in [11]; cells were scraped off in ice-cold PBS and lysed by the addition of 100 μl of lysis buffer [20 mM Tris/HCl (pH 7.5)/20% (v/v) glycerol/2 mM DTT/400 mM KCl] per 100 mm plate. Two freezing and thawing cycles were performed; protein extracts were then clarified at 4 °C in a Microfuge for 10 min and stored under liquid nitrogen.

The protein concentration of the extracts was measured by the Bio-Rad protein assay kit in accordance with the manufacturer's instructions.

Electrophoretic mobility-shift assay (EMSA)

Single-stranded (s.s.) oligonucleotides (1 pmol) were end-labelled with 40 μCi of [γ - ^{32}P]ATP (Amersham; specific radioactivity 3000 Ci/mmol) in the presence of 16 units of T4 polynucleotide kinase (Boehringer), at 37 °C for 30 min and then purified on polyacrylamide gels. The -13 to +44 double-stranded (d.s.) DNA fragment was released by digesting the pCAT-13+44 construct (in which CAT represents chloramphenicol acetyltransferase) [8] with *Hind*III and *Pst*I restriction enzymes. The fragment was purified from agarose gel with the QIAEX gel extraction kit (Qiagen), and the *Hind*III cleavage site was filled in by using the Klenow polymerase enzyme (Promega) in the presence of [α - ^{32}P]dCTP and [α - ^{32}P]dATP (Amersham; 40 μCi of each, specific radioactivity 3000 Ci/mmol). The reaction was incubated for 30 min at room temperature, then chased for 10 min with unlabelled dCTP and dATP; the labelled probe was finally purified on a G50 spin column. When the -13 to +44 fragment was used as an unlabelled competitor, the filling in with labelled dCTP and dATP was omitted.

For EMSA, appropriate amounts of protein were incubated on ice for 15 min in 18 μl of binding buffer [10 mM Tris/HCl (pH 7.5)/100 mM KCl/2% (w/v) Ficoll/6% (v/v) glycerol/1 mM DTT] with 2 μg of poly(dI)·poly(dC) (Pharmacia) as a non-specific competitor. After the addition of 5×10^4 c.p.m. of the labelled fragment, the reaction mixture was incubated at room temperature for 15 min. For competition experiments, unlabelled DNA fragments were added to the mixture during the first 15 min of incubation. The following d.s. oligonucleotides were used as competitors or probes in gel-retardation assays (only the top strand is shown): YY1, 5'-GTTTTGCGACAT-TTGCGACAC-3'; mutYY1, 5'-GTTTTGCGAGTTGGCG-ACAC-3'; NF-I, 5'-ATTGGCACTGACGCCAAT-3'.

The following s.s. oligonucleotide was used as competitor or probe in gel retardation assays: MF 0677, 5'-GGAGGTGGT-GGAGGGAGAGAAAAG-3'.

The DNA-protein complexes were resolved on 5% (w/v) polyacrylamide gels in 0.25 $\times$ TBE (22 mM Tris/borate/0.5 mM EDTA); electrophoresis was performed at room temperature at 10 mA. Gels were dried and autoradiographed with intensifying screens at -70 °C. When required, the incubation of the EMSA reactions with the YY1 Trans Cruz supershift antibody (Santa

Cruz Biotechnology) or with a non-immune serum was performed in accordance with the instructions of the manufacturer.

Purification procedure and cross-linking with UV

Nuclear extracts from bovine brains were loaded on a 150 ml DEAE column (DEAE-Sepharose fast flow; Pharmacia Biotech) in dialysis buffer containing 100 mM KCl at a flow rate of 1.2 ml/min. Retained proteins were eluted by raising the KCl concentration of the eluent successively to 250 and 500 mM; the 250 mM fraction, containing the activity forming the BI and BII complexes as assessed by EMSA, was dialysed in buffer A [40 mM Hepes (pH 7.4)/6 mM MgCl_2 /0.2 mM EDTA/20% (v/v) glycerol/1 mM DTT] [12] containing 200 mM KCl, then loaded on a 5 ml HiTrap heparin-Sepharose column (Pharmacia Biotech). The retained sample was eluted by a linear KCl gradient (from 0.2 to 1 M) on an FPLC apparatus (Pharmacia Biotech) at a flow rate of 5 ml/min.

The active fractions from the heparin-Sepharose column, eluted at a KCl concentration between 0.5 and 0.7 M, were pooled and dialysed in buffer Z [25 mM Hepes (pH 7.6)/100 mM KCl/12.5 mM MgCl_2 /1 mM DTT/20% (v/v) glycerol/0.1% (v/v) Nonidet P40] and loaded on a DNA affinity column constructed with the multimeric -13 to +44 oligonucleotide in accordance with a published procedure [13]. Retained proteins were eluted by a 14 ml linear KCl gradient (from 0.1 to 2 M). Active fractions were resolved by SDS/PAGE [12% (w/v) gel] and silver stained with a commercially available kit (Sigma).

For the UV cross-linking experiment, fraction 7 from the DNA affinity step was challenged with the -13 to +44 oligonucleotide and with a control probe from the human H ferritin promoter; the corresponding mixtures were irradiated for 5 min in a Bio-Rad UV cross-linker. The samples were finally resolved by SDS/PAGE [10% (w/v) gel] and autoradiographed.

Tryptic digestion, peptide purification and sequence analysis

Protein samples were digested with trypsin in 50 μl of 50 mM Tris/HCl, pH 8, at 37 °C for 24 h, with an enzyme-to-substrate ratio of 1:30 (w/w). The resulting peptides were fractionated directly by reverse-phase HPLC on a Supelcosil LC18DB column (Supelco) eluted with a linear gradient of acetonitrile from 2% to 80% in 0.1% trifluoroacetic acid over 65 min, at a flow rate of 0.2 ml/min. Fractions were collected manually and analysed by automated Edman degradation. Sequencing was performed by using a Perkin-Elmer-Applied Biosystems 477A pulsed-liquid protein sequencer equipped with a Perkin-Elmer-Applied Biosystems 120A apparatus for phenylthiohydantoin-amino acid identification.

Molecular cloning of Pur α cDNA

The cDNA encoding the full-length Pur α cDNA was cloned by screening a rat brain cDNA library (Stratagene) with the 5'- ^{32}P -labelled oligonucleotide PUR-F (CEINGE) as a probe, corresponding to the cDNA region encoding the N-terminal region of the mouse Pur α sequence, by standard procedures [14]. The PUR-F oligonucleotide sequence is 5'-ATGGCGGACCGAG-ACAGCCGGCAGCGAGCAGGGT-3'. Two positive clones were isolated, from which the pBluescript SK-Pur α plasmids were rescued by excision *in vivo* in accordance with the instructions of the manufacturer. The clones obtained were identical; one of them was entirely sequenced with the Sequenase kit (United States Biochemical). The full-length rat Pur α cDNA

from the selected plasmid was isolated by digestion with the *EcoRI* and *XhoI* restriction enzymes and subcloned in the pRc/cytomegalovirus (CMV) expression vector (Invitrogen) by standard cloning procedures [14].

Cell cultures and transfection experiments

Chinese hamster ovary (CHO) cells were grown in a humidified air/CO₂ (19:1) atmosphere at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum and 1% (w/v) antibiotics (HiClone).

Cell transfections were performed by the calcium phosphate method, as previously described [8]. Cells were plated 12 h before transfection at density of 3×10^5 cells per 60 mm dish; 4 μ g of a reporter plasmid, in which the -13 to +44 promoter region of the FE65 gene drives the transcription of the CAT gene [8], were co-transfected with different amounts of the YY1 expression vector (pCEP4-UCRBP, kindly provided by Dr. K. Ozato; containing the human YY1 cDNA under the control of the CMV promoter [15]) or the Pur α expression vector (pRC/CMV-Pur α , containing the rat Pur α cDNA under the control of the CMV promoter), or both. The total amount of transfected DNA (20 μ g) was kept constant with appropriate amounts of empty vectors. At 48 h after transfection, CAT assays were performed with 30 μ g of total cell extract from each sample by published procedures [8]. Relative CAT activity was measured as the amount of chloramphenicol converted into its acetylated forms. Western blot analysis of Pur α and YY1 proteins was performed by standard procedures [5] with anti-Pur α (gift from Dr. E. Johnson) and anti-YY1 monoclonal antibodies (gift from Dr. T. Shenk) at 1:2000 and 1:1000 dilutions respectively.

RESULTS

Transcription factor Pur α binds to the FE65 gene minimal promoter

We previously demonstrated that a short region of the FE65 TATA-less promoter, from nt -13 to nt +44, thus including the transcription start site, is able to drive significant transcription only in neural cells. This DNA region forms several DNA-protein complexes when incubated with nuclear extracts from different neural and non-neural cell lines [8]. To identify the proteins interacting with this region of the FE65 promoter we attempted their purification from bovine brain. The nuclear extract from bovine brain, analysed by EMSA with the -13 to +44 region as a probe, formed at least three specific complexes, named BI, BII and BIII (see Figure 1A, lane 2).

As described in the Materials and methods section, the proteins responsible for the formation of complexes BI and BII were purified by DEAE chromatography, followed by heparin-Sepharose chromatography and DNA-affinity chromatography. Figure 2 shows the EMSA obtained by using the fractions eluted from DNA-affinity chromatography. The fractions were analysed by SDS/PAGE (see Figure 3); this allowed us to observe that in fractions 5, 6 and 7, which showed the highest intensity in the shifted bands (see Figure 2), two main bands were present with molecular masses of approx. 38 and 40 kDa. Fraction 7 of the DNA-affinity chromatography was incubated with the specific -13 to +44 oligonucleotide probe or with an unrelated DNA fragment from the human H-ferritin promoter; the mixtures were cross-linked with UV and subjected to SDS/PAGE [12% (w/v) gel]. Only the sample containing the -13 to +44 oligonucleotide formed two covalent complexes with an apparent molecular mass of approx. 70 kDa: this size is in a good

Figure 1 DNA-protein complexes formed by the FE65 gene minimal promoter

(A) Nuclear extract from rat brain was analysed by gel-shift experiments to test its ability to interact with the -13 to +44 FE65 promoter region as described in the Materials and methods section. Lane 1, free probe; lane 2, no competitor; lane 3, extract preincubated with unlabelled -13 to +44 DNA fragment, 100-fold excess; lane 4, extract preincubated with unlabelled MF0677 s.s. oligonucleotide, 100-fold excess; lanes 5 and 6, extract preincubated with unlabelled YY1 oligonucleotide or with unlabelled mutated (mut.) YY1 oligonucleotide respectively, 100-fold excess; lanes 7 and 8, extract preincubated with anti-YY1 antiserum or with non-immune serum respectively. (B) Alignment of the nucleotide sequence encompassing the transcription start site of FE65 gene with various initiator elements (see [42]). The underlined nucleotides represent the transcription start sites.

agreement with the expected mass of the covalent DNA-protein complexes (see Figure 4).

Both 38 and 40 kDa proteins were electroblotted on a PVDF membrane and the excised bands were subjected to sequence analysis; however, the samples were not susceptible to Edman degradation, suggesting the presence of an N-terminal blocking group in both cases. Therefore fractions 5, 6 and 7 from DNA-affinity chromatography were desalted, concentrated and subjected to trypsin digestion for internal sequence determination. The peptide mixture obtained was separated by reverse-phase HPLC; some of the fractions obtained were analysed by auto-

Figure 2 Purification of transcription factors binding to the -13 to +44 FE65 promoter region

EMSA of fractions eluted from affinity chromatography with -13 to +44 DNA. Lane 1, free probe; lanes 2-15, fractions (Fr.) of DNA affinity chromatography eluted with a linear KCl gradient from 0.1 to 2 M.

mated Edman degradation (see Figure 5). This allowed us to obtain an unambiguous sequence of nine peptides, which was compared with the protein sequence databank. All the sequenced peptides are contained in the already known Pur α protein [16,17], suggesting that the two purified proteins of 40 and 38 kDa are different forms of the Pur α protein, probably a consequence of different post-translational modifications or maturation [18].

Pur α is a protein involved in both DNA replication [19] and transcription activation [20,21] and was isolated for the first time for its ability to bind an s.s. *cis* element, named MF0677 [16]. To

Figure 3 SDS/PAGE of DNA-affinity chromatography fractions

Lane 1, Size markers (molecular masses shown at the left, in kDa); lane 2, loaded proteins; lane 3, flow-through; lanes 4 to 17, fractions (Fr.) from DNA-affinity chromatography eluted with a linear KCl gradient from 0.1 to 2 M. The arrows indicate the migration of the 40 and 38 kDa proteins enriched in fractions 5, 6 and 7.

Figure 4 UV cross-linking of purified proteins from DNA-affinity chromatography

Fraction 7 from DNA-affinity chromatography was incubated with the 32 P-labelled -13 to +44 DNA fragment from the FE65 gene (lanes 1 and 2) or the 55 bp DNA fragment of the human ferritin gene promoter (lanes 3 and 4). After UV cross-linking in the presence or the absence of the purified proteins, the samples were analysed by SDS/PAGE. Lanes 1 and 3, free probes; lanes 2 and 4, proteins preincubated and cross-linked with -13 to +44 fragment or with an unrelated DNA fragment respectively.

Figure 5 HPLC analysis of the peptides resulting from tryptic digestion of the purified proteins

Fractions 5, 6 and 7 from DNA-affinity chromatography were combined and, after tryptic digestion, were analysed by HPLC as described in the Materials and methods section. The unambiguous amino acid sequences are reported on the corresponding peaks.

confirm that complexes BI and BII were due to the binding of Pur α to the -13 to +44 promoter region, competition experiments were performed by using as competitor the MF0677 s.s.

Figure 6 Competition experiments and cDNA over-expression confirm that Pur α factor binds to the -13 to +44 FE65 gene fragment

(A) Gel-shift experiments of rat brain nuclear extract incubated with MF0677 s.s. oligonucleotide. Lane 1, free probe; lane 2, uncompeted reaction; lanes 3–5, extract preincubated with increasing amounts (fold excesses) of unlabelled MF0677 oligonucleotide; lanes 6–8, extract preincubated with increasing amounts (fold excesses) of unlabelled -13 to +44 oligonucleotide; lane 9, extract preincubated with 300-fold excess unlabelled unrelated oligonucleotide. (B) Gel-shift experiment of nuclear extract from CHO cells transfected with CMV-Pur α expression vector, incubated with -13 to +44 oligonucleotide. Lane 1, free probe; lane 2, rat brain nuclear extract; lane 3, mock-transfected CHO cell extract; lanes 4 and 5, extracts from CHO cells transfected with 2 or 5 μ g of CMV-Pur α expression vector respectively.

oligonucleotide. As shown in Figure 1(A) (lane 4), the MF0677 s.s. oligonucleotide competed with the FE65 promoter -13 to +44 region for the formation of complexes BI and BII.

Similarly, by using the MF0677 s.s. oligonucleotide as a probe

we demonstrated that brain extracts were able to shift the migration of this s.s. oligonucleotide in EMSA experiments and that the binding of Pur α to this probe was efficiently competed for by the unlabelled d.s. -13 to +44 FE65 oligonucleotide (see Figure 6A).

The cDNA encoding the complete open reading frame of Pur α was cloned by screening a rat brain cDNA library with the PUR F oligonucleotide reported in the Materials and methods section as a probe; an expression vector was constructed by inserting this cDNA into the pRC/CMV plasmid, so that its transcription was under the control of the CMV promoter. This construct was transiently transfected in CHO cells, and the EMSA experiments performed with extracts from these cells are shown in Figure 6(B). Extracts from pRC/CMV-Pur α -transfected cells show the appearance of a BI complex identical with that observed with rat brain extracts.

Transcription factor YY1 binds to the FE65 gene minimal promoter

The FE65 gene promoter lacks a TATA element and shows, at the level of the transcription start site, an initiator consensus sequence. The analysis of this sequence showed that it is similar to various *cis* elements for different initiator binding proteins (see Figure 1B). Among the latter, the YY1 *cis* element is particularly similar. Competition experiments demonstrated that an oligonucleotide containing the YY1 recognition motif was able to compete for the formation of the BIII complex, whereas a mutant version of this oligonucleotide did not (see Figure 1A, lanes 5 and 6). TdT Inr [22], another *cis* element showing similarity to the FE65 promoter, does not compete for any of the complexes (results not shown). Furthermore the exposure of the rat brain nuclear extract to a polyclonal antibody directed against the YY1 protein specifically prevented the formation of the BIII complex, whereas an unrelated antibody had no effect (see Figure 1A, lanes 7 and 8).

YY1 and Pur α activate the FE65 gene promoter

The effects of YY1 and Pur α on transcription driven by the FE65 promoter were tested in CHO cells, in which its efficiency is very low compared with that observed in neuronal cells. Pur α and YY1 cDNA expression vectors were co-transfected with a CAT construct in which the CAT gene transcription is driven by the FE65 minimal promoter. As demonstrated in Figure 7, the overexpression of Pur α or YY1 cDNA species (see Western blot analyses shown in Figure 7D), increased approx. 3–4-fold the efficiency of the FE65 promoter. The transfection of large amounts of Pur α or YY1 expression constructs resulted in a decrease in CAT activity, probably as a consequence of squelching of factor(s) necessary for the transcription. This is in agreement with previous observations showing that high levels of Pur α or YY1 cause an inhibition of transcription [15,21]. The co-expression of Pur α and YY1 shows no apparent co-operation in regulating the FE65 minimal promoter activity. In fact, the co-transfection of Pur α and YY1 in CHO cells does not show any co-operative effect on transcription, which reaches a maximum 3-fold increase in relative CAT activity (see Figure 7C).

DISCUSSION

It was previously demonstrated that a short region of the FE65 TATA-less promoter, from nt -13 to nt +44, therefore including the transcription start site, is able to drive significant transcription in neural cells and forms several DNA-protein complexes with protein extracts from different cell lines.

Figure 7 FE65 minimal promoter efficiency in CHO cells transfected with Pur α and YY1 expression vectors

(A) The indicated amounts of CMV-Pur α expression vector were co-transfected with a plasmid carrying the CAT gene under the control of the -13 to +44 fragment of the FE65 gene promoter (open bars) or under the control of the RSV LTR promoter (filled bars), as described in the Materials and methods section. The reported fold increases in CAT activity, calculated as percentages of conversion of the substrate into its acetylated form, are the means $\pm$ S.D. for three independent experiments. (B) As (A), except that co-transfection was with indicated amounts of pCEP4-YY1 expression vector. (C) Co-transfection of the indicated amounts of pCEP4-YY1 expression vector with 0, 0.5, 1 or 5 μ g of CMV-Pur α expression vector and with 4 μ g of reporter gene construct. (D) Western blot analyses of Pur α and YY1 proteins in CHO cells transiently transfected with expression vectors. Lanes 1 and 3, mock-transfected cells; lanes 2 and 4, CHO cells transfected with 5 μ g of expression vectors; lanes 1 and 2, anti-Pur α antibody (1:2000 dilution); lanes 3 and 4, anti-YY1 antibody (1:1000 dilution).

We studied which factors are involved in the formation of the DNA-protein complexes on the FE65 minimal promoter in mammalian brain, and observed that two transcription factors, Pur α and YY1, bind to this promoter.

Pur α was isolated for the first time as an s.s. DNA-binding protein that interacts with purine-rich elements; one of these is located upstream of the P1 promoter of the *c-myc* gene [16], which is involved in DNA replication initiation. It was demonstrated that Pur α interacts with various gene promoters and in myelin basic protein gene it binds to a promoter element located in the -50 to -14 region upstream of the transcription start site and functions as a transcription activator [21]. Similarly, transcription of the quail clusterin gene is also activated by Pur α [23]. Pur α is developmentally regulated and shows significant tissue specificity. In fact, by measuring Pur α DNA-binding activity, it was observed that it is highly represented in mouse brain and is almost undetectable in mouse liver [24]. This is in agreement with

FE65 gene expression, which is abundant in several regions of the nervous system but is barely detectable in the liver [2].

Another level of the regulation of Pur α DNA-binding activity is represented by the availability of the molecule. It was demonstrated that Pur α binds to the hypophosphorylated form of the retinoblastoma protein (pRb) and that this protein-protein interaction prevents the binding of Pur α to s.s. DNA [11]. Several results indicate that pRb is involved in the development of the nervous system, and in fact high levels of the hypophosphorylated form of this molecule are associated with neuronal differentiation [25,26]. Therefore it can be speculated that in neuronal cells hypophosphorylated pRb would prevent the binding of Pur α to s.s. DNA. In contrast, it was clearly demonstrated, by using a d.s. probe, that a DNA-binding activity, thereafter identified as Pur α , increases during mouse brain development and is high in adult mouse brain but very low in adult liver [24]. Accordingly, our results indicate that the BI and

BII complexes generated by the binding of Pur α are very abundant in rat brain extracts (see Figures 1 and 6B), whereas the amounts are low in liver extracts (results not shown).

The s.s. and the d.s. DNA-binding activities of Pur α could be two different functional aspects of the same molecule, controlled by different regulatory mechanisms. The demonstration of a binding of Pur α to d.s. DNA, distinct from its s.s. DNA-binding activity, has until now been elusive. In fact, with the use of a d.s. probe the possibility cannot be excluded that Pur α functions as a helix-destabilizing factor, thus inducing the exposure of s.s. DNA regions. Our results show that the d.s. -13 to +44 element is able to compete for the binding of Pur α to the s.s. MF0677 probe (see Figure 6A). This competition is about one-tenth as efficient as that observed with the unlabelled MF0677 s.s. oligonucleotide or with the melted -13 to +44 fragment (results not shown). In any case it is unlikely that the d.s. -13 to +44 fragment preparation used for the gel-shift experiments contains large amounts of s.s. molecules because these DNA fragments were generated by restriction enzyme cleavage of a plasmid and not by the annealing of synthetic oligonucleotides.

The second factor able to bind to the FE65 promoter is YY1. This protein shows the unique feature of activating, repressing or controlling the correct initiation of transcription, depending on the promoter context where its *cis* element is located [27,28]. The polyfunctional activity of this factor is under the control of many regulatory mechanisms, so it could also mediate cell- and tissue-specific gene transcription either positively or negatively. The first mechanism by which YY1's function can be regulated is physical interaction with other proteins such as Sp1 [29], c-Myc [30], B23 [31], p300 [32], TAF_{II}55 [33], TFIIB [34] and YAF2 [35]. The second possibility is represented by the ability of YY1 to induce DNA bending; in this case the specific position of the YY1 *cis* element in the context of other binding sequences could result in a modulation of the DNA binding of other transcription factors [36]. A third mechanism might result from the possible competition between YY1 and other transcription factors for overlapping *cis* elements [37–41]. In this case the availability of YY1 molecules competent for the binding to DNA would have a key regulatory role. This availability could be primarily related to the amount of YY1 present in the cell. A tissue-specific regulation of the YY1 level was not demonstrated, although a detailed quantitative analysis of this protein in various cellular compartments of the nervous system showed a different concentration in different cell types. For example, during the differentiation of chick myoblasts the amount of YY1 decreases [39]. In the case of the FE65 minimal promoter, YY1 seems to interact with the initiator sequence, so it activates transcription by functioning as an initiator-binding protein; however, interaction with other proteins, effects of a YY1-induced bending and/or competition with inhibitory factors cannot be excluded.

The possibility of simultaneous binding of two different *cis* elements of Pur α and YY1 to the FE65 minimal promoter is not supported by the present results. In fact, exposure of brain nuclear extracts to an anti-YY1 antibody and competition with an oligonucleotide containing the YY1 *cis* element erase only complex BIII, leaving complexes BI and BII unaffected. In contrast, competition with the s.s. MF0677 oligonucleotide, which binds Pur α , does not affect the intensity of the BIII complex. This means that none of the three complexes observed is the result of the contemporary binding of the two factors. Therefore the results of over-expression of Pur α and/or YY1 in CHO cells suggest that two different mechanisms of FE65 gene activation exist, which could be cell-type specific and could be sensitive to different regulatory pathways. This is in agreement with the observation that PC12 cell nuclear extracts form the Pur

α -specific complexes ([8], and G. Minopoli, R. Faraonio, N. Zambrano and T. Russo, unpublished work) and this parallels a significant activity of the FE65 minimal promoter in these cells, whereas in Rat2 fibroblasts, in which the efficiency of the FE65 gene promoter is poor, the nuclear extracts do not form Pur α complexes with the -13 to +44 probe [8]. In contrast, in N-TERA2 cells, differentiation towards the neuronal phenotype is accompanied by activation of the FE65 promoter [8] and by a significant increase in the YY1-specific complex (G. Minopoli, N. Zambrano and T. Russo, unpublished work).

An important point to be explored is the relationship between Pur α and YY1 with the factors interacting with upstream elements of the FE65 gene promoter. One of these functions as a non-cell-specific enhancer and binds Sp1 or an Sp1-like factor [8]; other *cis* elements are currently under examination to evaluate their contribution to the efficiency of the promoter and the role of Pur α and YY1 in the regulation of their functions.

We thank K. Ozato and T. Shenk for providing us with the plasmid pCEP4-UCRBP and with the anti-YY1 monoclonal antibody; E. Johnson for the anti-Pur α antibody; and G. Marino for helpful discussion. This work was supported by grants from Telethon (grant no. E522), CNR, MURST 40% and 60%.

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Received 8 May 1997/16 July 1997; accepted 23 July 1997