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The Leu-Enkephalin-Encoding Sequence DNA-Binding Factor (LEF) Is the Transcription Factor YY1

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The Leu-enkephalin-encoding sequence DNA-binding factor (LEF) with high affinity for the Leu-enkephalin-encoding sequences in the prodynorphin and proenkephalin genes has earlier been identified. This factor is composed of three subunits of about 60, 70 (the major DNA-binding subunit), and 95 kDa, respectively. Estimated molecular mass, sequence specificity of DNA-binding, and supershift/inhibition with specific antibodies in a band shift assay showed that the DNA-binding subunit of LEF is identical to the multifunctional transcription factor YY1. However, an antibody against the C-terminus of YY1 distinguished the YY1 complexes with a Leu-enkephalin-encoding sequence and canonical YY1 binding site oligonucleotides, suggesting different protein conformations in complexes with these two DNA fragments. © 1997 Academic Press

It was earlier reported that short, conserved and repetitive sequences encoding the Leu-enkephalin fragments of α-neoendorphin and dynorphins A and B in the prodynorphin gene (PD), and Leu-enkephalin in the proenkephalin gene (PE), represent targets for a specific DNA binding factor, which was named Leu-enkephalin-encoding sequence DNA binding factor (LEF) (1). Whereas LEF demonstrated high affinity for the Leu-enkephalin-encoding sequences, it did not bind well to the Met-enkephalin-encoding sequences from the PE and proopiomelanocortin (POMC) genes. LEF is composed of a major 70 kDa DNA-binding subunit and two additional subunits of about 60 kDa and 95 kDa, respectively. Expression of the factor is developmentally regulated in rats, with the highest levels recorded in brain from late embryos. LEF inhibited the transcription of reporter genes apparently through the binding to the Leu-enkephalin encoding sequence inserted between a promoter and the transcription initiation site (1). Very recently, a new prodynorphin transcript with the initiation site located in exon 4, upstream of the Leu-enkephalin encoding sequences, was identified in fetal brain and tumor cell lines (2). The proximity of the initiation site for the truncated prodynorphin transcript with the potential LEF binding sites, suggests the involvement of LEF in the regulation of PD gene transcription.

During characterization, it was considered possible that LEF or its subunits might relate or be identical to a previously characterized factor. Based on results to be described here, a likely candidate was Yin-Yang 1 (YY1; also known as δ, NF-E1, or UCRBP), a zinc finger transcription factor belonging to the GLI-Krüppel family of nuclear proteins (3-6). Depending upon the promoter context, YY1 can either repress transcription of genes, c-*fos* (7), casein (8), globin (5,9) and many other cellular and viral genes (3-6), or activate transcription, c-*myc* (10) and p53 (11). YY1 has also been found to direct and initiate transcription when positioned at transcription initiation sites (4,6,12). Putative YY1 binding sites are located in enhancer/promoter regions, introns and 3′ flanking sequences of different genes (4,6,9).

In this study, LEF was purified to apparent homogeneity, its major DNA-binding subunit identified as YY1 and the Leu-enkephalin encoding sequences found to differ in binding properties from the canonical YY1 binding sites.

MATERIALS AND METHODS

Oligonucleotides and proteins. Oligonucleotides were synthesized on a Gene Assembler Plus Synthesizer (Pharmacia, Uppsala, Sweden) and purified by gel electrophoresis or were obtained commercially from Santa Cruz Biotechnology, Santa Cruz, CA (oligonucleotides with wild type (wt) and mutant (m) YY1 consensus binding site). The plus strand sequences of oligonucleotides used are shown in Table 1; minus strands were either complementary to the plus strands (wtYY1, mYY1, oligonucleotides with YY1 binding sites corresponding to the LINE-1 transposon (YY1L) (13) and the casein promoter (YY1C) (8), as well as short wt-dynorphin B-encoding (sDB)

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TABLE 1

Sequences of Oligonucleotides Used in a Band Shift Assay and Their Relative Binding Affinities

Oligonucleotide[a]	Sequence[b]	Affinity[c]
wtDB	GCAGAAGCGCTATGGCGGTTTTCTCCGGCGCCAGTTC	1
sDB	AAGCGCTATGGCGGTTTTCTCCGGCGC	0.8
mDB	GCAGAAGCGCTAT*CTTC*GTTTTCTCCGGCGCCAGTTC	<0.001
DA	GGTCAAACGCTATGGGGGCTTTTTGCGCAAATACC	0.2
ME3	CGCCAAGCGGTATGGGGGCTTCATGAAGAAGGATG	0.05
wtYY1	ACCAGCCGCCAAGATGGCCGCGGAGCG	1
mYY1	ACCAGCCGCCAAGA*TAA*TCGCGGAGCG	<0.001
YY1C	CATGATTAGAAAATGGTTTCTTTCTAT	nd
YY1L	GGAGGAGCCAAGATGGCCGAATAGGAA	nd

[a] Human PD gene fragments (1) including sequences encoding human Leu-enkephalin of wild type dynorphin B (wtDB), dynorphin A (DA), a human PE gene fragment including the nucleotide sequence encoding Met-enkephalin (ME3; the third from the 5′-end) (1), as well as a short dynorphin B-encoding oligonucleotide (sDB), a mutant dynorphin B-encoding oligonucleotide (mDB), and oligonucleotides with wild type and mutant YY1 consensus binding site (4) (wtYY1 and mYY1, respectively), with a YY1 binding site in the casein promoter (8; YY1C) and LINE-1 transposon (13; YY1L), were used.

[b] The Leu-enkephalin-encoding sequence (in wtDB) is overlined and YY1 core binding sequences are underlined. Mutant nucleotides are shown in italics.

[c] Concentrations of oligonucleotides inhibiting the binding of labelled YY1 by 50% were used for calculations of relative affinities. nd, not determined.

and m-dynorphin B-encoding (mDB) oligonucleotides) or their complementarity regions were extended by 1-4 nucleotides from the 5′-end and recessed by 1-3 nucleotides from the 3′-end (1). Double-stranded m1- and m2-dynorphin A-encoding oligonucleotides (1) were used for preparation of a precolumn and a DNA-affinity column, respectively, as described by Kadonaga and Tjian (14).

Preparation of nuclear extracts. Nuclear extract was prepared from brain or whole 17-day-old embryos of Sprague-Dawley rats using a protocol adapted (1) from Dignam *et al.* (15). Briefly, tissues were homogenized in Dignam's buffer A, supplemented with protease inhibitors. The homogenate was centrifuged for 10 min at 2500 g, the pellet was extracted in buffer C, supplemented with 0.2% NP-40 and protease inhibitors, centrifuged at 20,000×g for 10 min × 2, and the resulting supernatant, designated the "nuclear extract", was used for LEF purification or kept at −80°C until studied.

Band shift assay. For an assay performed essentially as described previously (1,16), 1 μg of nuclear extract protein or 1-4 ng of purified LEF, as well as 0.5 μg poly[dI-dC]-poly[dI-dC] were incubated in 20 μl of reaction mixture. Affinity-purified rabbit polyclonal antibodies H414, C20 and p50 (Santa Cruz Biotechnology) against full length human YY1, its carboxy terminus (amino acids 395-414) or the p50 subunit of NF-κB, respectively, were used in supershift/depletion experiments. The antibodies were incubated with protein extract or purified LEF for 1 h on ice before electrophoresis. For neutralization, C20 antibody (0.1 μg IgG) was preincubated with blocking peptide (0.4 μg; Santa Cruz Biotechnology) for 30 min on ice before incubation with extract.

LEF purification. Solutions used for purification contained 0.5 mM $MgCl_2$ and were supplemented with protease and phosphatase inhibitors (17). Nuclear extract obtained from 300 g rat embryos was diluted to 0.1 M NaCl and subjected to DNA-cellulose chromatography as described (17). The eluted fraction with LEF activity identified by band shift assay was dialyzed to a final concentration of 0.1 M NaCl and applied to the pre-column and DNA-affinity column connected in tandem (17). LEF activity was eluted from the affinity column with N300 buffer (17) containing 0.3 M NaCl. The eluate was diluted to a final concentration of 0.1 M NaCl, reloaded onto the DNA-affinity column for a second round of purification, thereafter dialyzed and kept at −20°C (17). To test purity, 10-20 ng of affinity-purified LEF was loaded onto a 10% SDS-polyacrylamide precast gel (Novex, San Diego, CA). Proteins were visualized by silver staining with a kit from Pierce Chemical Company (Rockford, Il.).

Analysis of purified LEF. Affinity-purified LEF (10-20 ng) was incubated with nonlabelled wtDB or mDB oligonucleotide (50 ng) in the presence of labelled wtDB oligonucleotide, under conditions described for the band shift assay (concentration of bovine serum albumin was reduced to 200 ng in 20 μl), followed by resolution on a native 4% polyacrylamide gel. Nonlabelled oligonucleotides were used to shift the protein on a gel. Labelled wtDB oligonucleotide was used as a marker. The bands corresponding to the upper LEF-DNA complex (see Results) were cut out from the gel, the shifted proteins were resolved by 10% SDS-polyacrylamide gel electrophoresis and identified by silver staining. About 0.5 ng of shifted protein could be visualized on the gel.

RESULTS AND DISCUSSION

Isolation of LEF DNA-binding activity. LEF activity was purified from 17-day-old rat embryo nuclear extract by DNA-cellulose column chromatography followed by two rounds of DNA-affinity chromatography (Fig. 1, lanes 1-3). The highly purified material contained a major polypeptide with an apparent molecular weight of 67 kDa (lane 3). This polypeptide was shifted by the LEF target wtDB oligonucleotide (lane 5), but not the mDB oligonucleotide (lane 4), upon electrophoresis on a native gel. Additional bands in the range of 70-80 kDa (lanes 4-5) appeared on the SDS-polyacrylamide gel, probably due to contamination by bovine serum albumin present in the incubation medium.

Identification of LEF as YY1. Examination of molecular sizes and target sequences of known DNA-binding factors brought our attention to the 68 kDa nuclear factor YY1, since its core binding sequence CCAT is present, in reverse orientation, in LEF binding sites (Table 1). To determine whether the DNA-binding sub-

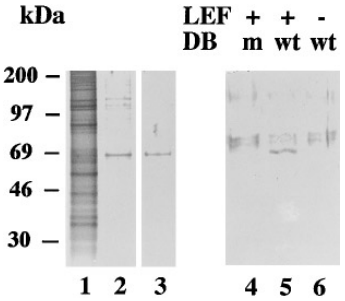

FIG. 1. Visualization of purified LEF protein by silver staining of a SDS-polyacrylamide gel. Molecular sizes of markers are indicated to the left. Lane 1 represents eluate from DNA-cellulose column chromatography and lanes 2 and 3 represent eluates from the first and the second rounds of affinity column chromatography, respectively. Affinity purified protein was incubated with labelled DB-encoding oligonucleotide used as a marker in the presence of nonlabelled wtDB (for protein shift) or mDB oligonucleotide and reaction products were separated on a native polyacrylamide gel (not shown; see Materials and Methods). The wtDB-oligonucleotide-LEF complex and the corresponding area from the mDB/LEF lane were cut out from the native gel, resolved by SDS-polyacrylamide gel electrophoresis and silver stained (lanes 4-6). Protein shifted by the wtDB oligonucleotide could be observed on the gel (lane 5), but there was no protein band shifted by mDB (lane 4).

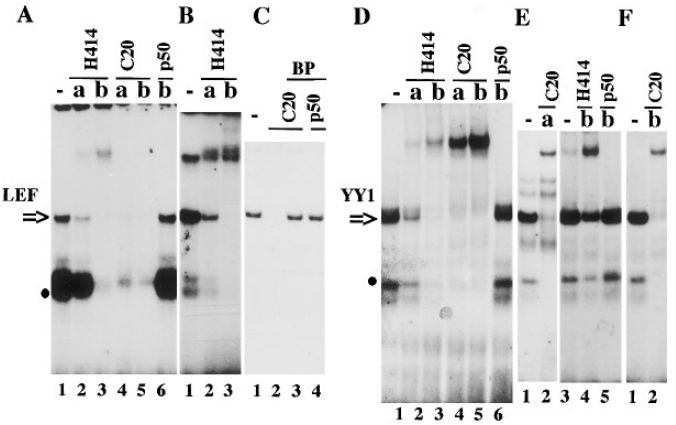

FIG. 3. Incubation of LEF with anti-YY1-antibodies H414 and C20 causes a supershift or inhibition in a band shift assay. Purified LEF (A,D) or nuclear extract of rat embryonal brain (B,C,E,F) was incubated with 0.1 (a, and lanes 2-4 in C) or 0.3 (b) μg of anti-YY1- or control anti-p50-antibodies in the presence (C, lanes 3,4) or absence of blocking peptide (BP), prior to the addition of labelled wtDB (A,B), short DB (C), wtYY1 (D), YY1C (E) or YY1L (F) oligonucleotide. The arrow and the closed circle indicate the positions of the retarded complexes.

unit of LEF is actually YY1 we tested oligonucleotides containing the wild type YY1 consensus binding sequence (wtYY1; Fig. 2C and 3D), the YY1 binding site in the casein promoter (YY1C; Fig. 3E) and in the LINE-1 transposon (YY1L; Fig. 3F) as labelled probes. Incubation of labelled wtDB and YY1 oligonucleotides with nuclear extract from rat embryonal brain produced retarded complexes with identical mobility (Fig. 2, lane 1 in A and C; Fig. 3, lane 1 in B,C,E,F). In addition to these complexes, faster migrating complexes were detected with purified LEF (Fig. 2B, lane 1; Fig. 3, lane 1 in A and D). The YY1 oligonucleotides were also used as competitors of LEF-oligonucleotide complex formation and found to inhibit the formation of the upper and lower protein-DNA complexes to the same extent as the wtDB oligonucleotide (Fig. 2A,B, and lanes 1-7 in C). However, the mYY1 and mDB oligonucleotides did not block the formation of complexes (Fig. 2A,B). Thus, LEF and YY1 demonstrated indistinguishable binding specificity.

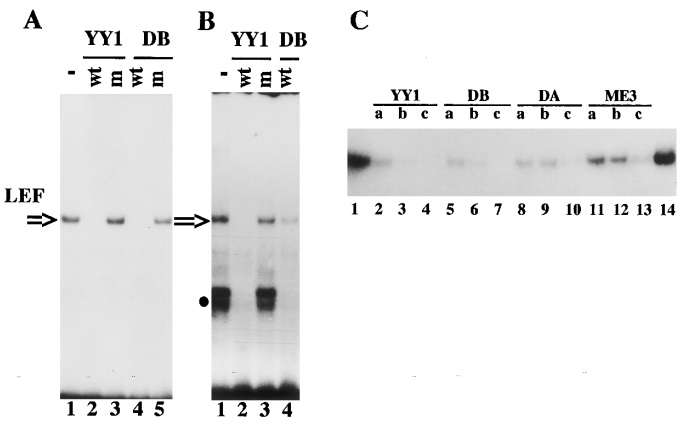

FIG. 2. Competition of LEF binding activity in a band shift assay with wtYY1, mYY1, wtDB, mDB, and ME3 oligonucleotides. Nuclear extract from rat embryonal brain (A,C) or purified LEF (B) was incubated with wtDB (A,B) or wtYY1 (C) oligonucleotides used as labelled probes. Competitors were used in concentrations either of 20 ng in 20 μl (A,B) or in concentrations equimolar to 1 ng (a), 5 ng (b) and 25 ng (c) of wtYY1 in 20 μl of reaction mixture (C). The arrow indicates the position of the LEF complex. The closed circle shows the position of the lower specific complex formed by purified protein.

Specific anti-YY1 H414 and C20 antibodies used in a band shift assay confirmed that LEF is indeed YY1 (Fig. 3). Purified protein (A,D) or nuclear extract from rat embryonal brain (B,C,E,F) were used in these experiments. The H414 antibody specifically supershifted LEF and YY1 complexes with labelled wtDB (lanes 2,3 in A,B) or wtYY1 (lanes 2,3 in D and lane 4 in E) oligonucleotides. Interestingly, the effects of the C20 antibody which had been raised against the C-terminus of YY1 depended on the type of labelled probe used. The antibody inhibited the complex formation with labelled wtDB (lanes 4,5 in A) and the short dynorphin B-encoding oligonucleotide (lane 2 in C). In contrast, supershifted complexes were observed with all three labelled YY1-related oligonucleotides, wtYY1 (D, lanes 4,5), YY1C (E, lane 2) and YY1L (F, lane 2). C20 antibody preincubated with a blocking peptide was unable to inhibit LEF-DNA complex formation (C, compare lanes 2 and 3). An antibody against the p50 subunit of NF-κB used for comparison had no effect on complex formation (lane 6 in A and D; lane 4 in C; and lane 5 in E). These

results indicate that LEF and YY1 have the same antigenic determinants. The C20 antibody inhibited complex formation with the DB oligonucleotide which suggests that the C-terminus of the protein is involved in binding to this oligonucleotide. When the protein binds to the canonical YY1 oligonucleotides, there is probably formation of a ternary DNA-YY1-C20 antibody complex registered as a supershifted band on the gel.

The upper and lower complexes obtained with purified LEF demonstrated the same binding specificities with DNA binding activity completely supershifted or inhibited by YY1-specific antibodies (Fig. 2 and 3). Casein (2 μg in 20 μl) which can activate DNA-binding factors (18), substantially promoted the formation of the upper complex as compared with the lower complex (data not shown). These observations suggest that the lower complex may arise by dissociation of the upper complex or that the upper complex in nuclear extracts is stabilized by additional protein(s) separated during LEF purification. Partial proteolysis of the upper complex can, probably, be ruled out since only one protein band corresponding to LEF was observed on a SDS-polyacrylamide gel (Fig. 1, lane 3).

Analysis of the LEF/YY1 binding site. LEF demonstrated the highest affinity for binding to the dynorphin B-encoding fragment, and bound less efficiently to the other Leu-enkephalin-encoding sequences of the PD and PE genes and did not form a complex with the Met-enkephalin-encoding sequences in the PE (the 3′-terminal copy) and POMC genes (1). These Met-enkephalin-encoding sequences do not have the YY1 target core sequence. Several other Met-enkephalin-encoding sequences, particularly the third sequence from the 5′-end (ME3) in the PE gene have the YY1 target core sequence CCAT in the reversed orientation (Table 1). However, the affinity of the ME3 oligonucleotide for binding to LEF/YY1 is much lower than that of the oligonucleotides coding for Leu-enkephalin (Table 1; Fig. 2C, compare lanes 11-13 with lanes 5-7, and 8-10). Therefore, the entire Leu-enkephalin encoding sequence, and not only the YY1 core sequence, seems to be involved in binding. This is consistent with the methylation interference analysis showing that the LEF binding sequence practically coincides with the 15 bp Leu-enkephalin-encoding sequence and additional GC bases from the 5′ end of the plus strand (1). YY1 has four C2H2 zinc fingers, each of them apparently contacting 3-5 bp, and consequently YY1 recognizes binding sites comprised of at least 12 bp (4-6). However, YY1 binding sites, displaying considerable heterogeneity, contain only 3-4 invariant bp, namely 5′-CAT-3′ or 5′-CCAT-3′, flanked by variable regions (4-6). Flexibility of DNA recognition by YY1 along with high affinity binding to 12-20 bp target sequences may be related to its ability either to activate or repress transcription via distinct activatory or inhibitory binding element (4-6).

TABLE 2

Comparison of the Dynorphin B-Encoding Sequence (in Reversed Orientation) with the Repressor and Activator YY1 Consensus Sequences (4), Respectively (Identical (|) and Matching (·) Nucleotides Are Shown)

Repressor	C C A T N T T N N N T/A
	| | | | · . . . |
wtDB	C C G C C A T A G C G C T T C
	| | | | | | · | |
Activator	C G G C C A T C T T G N C T C/G

In analyzing the sequences flanking the YY1 core site 5′-CCAT-3′, Shrivastava and Calame identified separate consensus gene sequences at which YY1 acts as an activator, as opposed to the sites at which YY1 acts as a repressor (4). YY1 binding sites in the LINE-1 transposon is representative of the activator sequence (4,13), whereas YY1 binding sites in the casein promoter (4,8) mediate repressor effects of this factor. The potential YY1 sites, located within the dynorphin B-encoding sequences of the PD gene, match the repressor consensus sequence slightly better (2 mismatches; Table 2) than the activator consensus sequence (5 mismatches). In accordance with this observation, LEF inhibited the transcription of reporter genes in artificial gene constructs where the dynorphin B-encoding DNA fragment had been inserted between the transcription initiation site and the coding region of the reporter genes (1).

High levels of LEF/YY1 were registered in the embryonal or newborn animals with the highest levels in embryonal brain (1). The levels generally decrease during development but are still high in the cerebellum and pituitary of adult animals (not shown). In adult brain, YY1 may repress the c-*fos* promoter (7) and activate the dopamine β-hydroxylase gene (19), but little is presently known about other putative targets and possible regulation of YY1 RNA or protein levels. The functional interplay between YY1 and AP-1, as well as YY1 and the ATF/CREB family of transcription factors, however, suggests the importance of this factor in the regulation of a number of processes in adult brain, particularly associated with long-term plastic changes.

In summary, several lines of evidence support the conclusion that the nuclear protein, previously referred to as LEF (1), which may play a role in the regulation of PD gene transcription, is identical to the transcription factor YY1. However, the conformation of the LEF/YY1 complex with the Leu-enkephalin-encoding sequence differs from those with the canonical YY1 binding sites irrespective of whether they activate or repress transcription.

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