Characterization of the mouse dynamin I gene promoter and identification of sequences that direct expression in neuronal cells

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Dynamin I is expressed at high levels in brain and its expression is regulated during the developmental stages of brain. To elucidate the molecular mechanism by which the expression is tissue-specifically regulated, we cloned the 5'-flanking region of the mouse dynamin I gene and determined the nucleotide sequence of 1036 bases upstream from the translation start site. Transient transfection studies with a chloramphenicol acetyltransferase reporter gene in neuroblastoma NS20Y and Lewis lung cells demonstrated that the 5'-flanking region has a cell-type-specific promoter activity. Deletion analyses demonstrated that the minimal promoter activity was detected in the proximal region 195 bp upstream of the translation initiation codon (–90 to +105). The minimal promoter was embedded in a GC-rich region (75 % GC content), in which an Sp1-binding motif and a

nuclear factor (NF)- κ B-like element (NE-1) were found, but it lacked TATA and CAAT boxes. Mutational analysis and electrophoretic mobility-shift assay analysis revealed that Sp1 binds to the Sp1 site and that this element is critical for the promoter activity of the dynamin I gene. We found that the NE-1 sequence is required for the expression of the dynamin I gene but NEBP (NE-1-binding protein), which binds to the NE-1 sequence, is not NF- κ B. We also found that one base in the NE-1 sequence (the underlined G residue in GGGATTCGCGGA) is critical for binding specificity to discriminate between NEBP and NF- κ B. By UV cross-linking analysis, we found that NEBP is an approx. 104 kDa nuclear protein.

Key words: dynamin I, nuclear factor κ B, promoter, Sp1.

INTRODUCTION

Dynamin is a 100 kDa GTPase required for the late stage in receptor-mediated endocytosis and for the recycling of synaptic vesicles in neurons (reviewed in [1–5]). It is believed to have a direct role in detaching clathrin-coated vesicles from the plasma membrane [6,7] but it has recently been proposed to have a role as a switch to regulate the endocytic step [8]. So far, at least three different dynamin genes have been found with different expression patterns and possibly different functions. The isoform identified originally, now referred to as dynamin I, is expressed at high levels in brain [9], where it might be dedicated to the recycling of synaptic vesicles [10]. Dynamin II is expressed ubiquitously [11], most probably promoting receptor-mediated endocytosis. Dynamin III is primarily expressed in the brain, lung and testis [12]; its function is not yet known.

Several papers have proposed that dynamin has a distinct role in the activation of MAP (mitogen-activated protein) kinase other than receptor endocytosis. Ligand-induced activation of MAP kinase [13,14], by both receptor tyrosine kinases and G-protein-coupled receptors [15,16], is also specifically inhibited by dominant-negative mutant dynamin. These observations have been interpreted to indicate that the endocytosis of a wide variety of signalling receptors is a general requirement for receptor-mediated signal transduction via mitogenic kinase cascades. However, Kranenburg et al. [17] have suggested that receptor internalization is not critical for the activation of MAP kinase and that the activation of MAP kinase by MAP kinase/ERK kinase (MEK) is regulated by dynamin.

Although the structure and function of the various members of the dynamin family have been studied extensively [18,19], there is little information on their expression mechanism. Early experiments demonstrated that dynamin I is up-regulated during rat brain development. Faure et al. [20] and Cook et al. [12] showed that dynamin I is present only at very low levels before embryonic day 16; thereafter its expression increases steadily, with adult levels being attained by postnatal day 23. Through the use of anti-sense oligonucleotides against dynamin I, they also have shown that this up-regulation is necessary for neurite formation [21]. These reports suggested that dynamin I gene expression is important for the development of brain; however, the regulation mechanism is not yet known.

In the present paper, to examine the molecular mechanism by which the expression of dynamin I is cell-type-specifically regulated, we cloned and characterized the promoter of the mouse dynamin I gene. To define the regions responsible for the cell-type-specific promoter activity, we performed deletion and site-directed mutagenesis analyses. From these studies we showed that an Sp1-binding site spanning –13 to –4 bp and a nuclear factor (NF)- κ B-like element (NE-1) sequence at –75 to –64 bp are critical for the constitutive expression of the dynamin I gene in mouse NS20Y neuroblastoma cells. We observed that the transcription factor Sp1 binds to the GC box and an approx. 104 kDa NE-1-binding protein (NEBP) binds to the NE-1 sequence. The NE-1 core sequence differs in a few nucleotides from a consensus NF- κ B-binding sequence; we also defined the sequence requirement that is critical for binding specificity to discriminate between NEBP and NF- κ B.

Abbreviations used: EMSA, electrophoretic mobility-shift assay; CAT, chloramphenicol acetyltransferase; NE-1, NF- κ B-like element; NEBP, NE-1-binding protein; NF- κ B, nuclear factor κ B.

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EXPERIMENTAL

Cloning of the 5'-flanking region of the mouse dynamin I gene

A 129SvJ mouse genomic library (Stratagene) was screened with a double-stranded cDNA probe spanning 147 bp from the start codon of the mouse dynamin I cDNA. The probe was labelled with [α - 32 P]dCTP with the High prime labelling system (Boehringer Mannheim). Bacteriophages were grown and transferred to nylon membrane. After cross-linking with UV, the filters were prehybridized in $6 \times$ SSC [$1 \times$ SSC is 150 mM NaCl/15 mM sodium citrate (pH 7.0)]/5 $\times$ Denhardt's solution/1% (w/v) SDS/50% (v/v) formamide at 42 °C for 1 h. Filters were hybridized with probe for 16 h. The filters were washed to a final stringency of $0.1 \times$ SSC/0.1% SDS at 55 °C. A 6 kb fragment double-digested with *NotI* and *XhoI* was subcloned into pBlue-script SK(−) and named pBS-dynIP. The sequences of pBS-dynIP were determined by dideoxy sequencing with Sequenase 2.0 (United States Biochemical Corporation) and adenosine 5'-[α - 32 S]thio]triphosphate.

Primer extension analysis

A 36-base oligonucleotide (EP) complementary to the coding strand at positions +70 to +105 was end-labelled with [γ - 32 P]ATP by T4 polynucleotide kinase. The 32 P-labelled probe was annealed to 10 μ g of total RNA from mouse neuroblastoma NS20Y cells at 30 °C for 16 h in buffer containing 80% (v/v) formamide, 40 mM Pipes (pH 6.4), 400 mM NaCl and 1 mM EDTA (pH 8.0) and extended by M-MuLV reverse transcriptase (Boehringer Mannheim). The extension products were analysed on an 6% (w/v) sequencing gel [89 mM Tris/HCl (pH 8.0)/89 mM boric acid/2 mM EDTA/8 M urea/5.7% (w/v) acrylamide/0.3% *N,N'*-methylene-bisacrylamide] along with a dideoxy DNA sequencing reaction of pBS-dynIP, with EP as the primer.

Cell culture

NS20Y, NIH 3T3 and Hepa1C1C7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum. Lewis lung and 70Z/3 cells were maintained in RPMI 1640 medium containing 10% (v/v) fetal bovine serum. All culture media contained 100 units of penicillin and 100 μ g/ml streptomycin.

Reporter plasmid construction and transient transfection procedure

PCR was used to generate the dynamin I sequences in plasmids pCATIP931 (in which CAT stands for chloramphenicol acetyltransferase) and 5' serial deletion constructs. To construct pCATIP931, 5'-primer oligonucleotide containing dynamin I sequences identical with positions −931 to −912 and a *HindIII* linker (5p) was used. The 3'-primer oligonucleotide used in all reactions was complementary to the dynamin-I-coding strand nt +86 to +105 and included an *SalI* linker (3p). pBS-dynIP was used as the template. PCRs were run with *Pfu* DNA polymerase (Stratagene) for 25 amplification cycles. The products were cut with *HindIII* and *SalI*, then subcloned into pCAT-Basic (Promega). All of the deletion constructs were made by the same methods; the constructs were named for their 5' end of the dynamin I promoter sequences (pCATIP931 to pCATIP0). The control plasmid pCMV β (Clontech) contained the cytomegalovirus promoter fused to the β -galactosidase gene. The plasmids pCATIP30mSp1 and pCATIP90mNE-1 are constructs in which the Sp1-binding site and NE-1 sequence were mutated

from pCATIP30 and pCATIP90 respectively. These constructs were generated by the overlap extension procedure [22]. They were transfected into NS20Y and other cells by means of Eugene 6 (Boehringer Mannheim). pCAT-Basic (2 μ g) and dynamin I-CAT reporter constructs were transfected with pCMV β into the cells in 10 cm dishes. Cells were harvested 48 h after transfection; β -galactosidase and CAT activities were measured.

CAT and β -galactosidase assays

To prepare cell extracts for assay for CAT and β -galactosidase expression, cells were scraped and centrifuged at 4 °C for 5 min. The cell pellet was resuspended in 150 μ l of 0.25 M Tris/HCl, pH 7.9, and then subjected to three freeze-thaw cycles. Cell debris was centrifuged at 4 °C for 5 min. The resulting supernatant was removed and used directly in the assays. The β -galactosidase assay was performed with chlorophenol red- β -D-galactopyranoside (CPRG; Boehringer Mannheim) and used to normalize CAT results. β -Galactosidase activity was measured with 10 μ l of cell extract in a reaction mixture consisting of 2.5 mM CPRG and 1.25 mM MgCl_2 . After incubation for 0.5–1 h at 37 °C, the reaction was stopped by the addition of 3 mM ZnCl_2 ; the absorbance of the reaction product was measured at 574 nm. CAT activity was determined with 50–100 μ l of cell extract in the presence of acetyl-CoA and [14 C]chloramphenicol in a total volume of 150 μ l. The mixture was incubated at 37 °C for 2 h, extracted with ethyl acetate and dried in a vacuum desiccator. Acetylated and non-acetylated forms of [14 C]-chloramphenicol were resuspended in 20 μ l of ethyl acetate and separated by TLC for 30 min at room temperature with chloroform/methanol (97:3, v/v) as the mobile phase. The percentage conversion of chloramphenicol into its acetylated forms was determined with a PhosphorImager.

Preparation of nuclear extracts and electrophoretic mobility-shift assay (EMSA)

To prepare the nuclear extracts, 1.0×10^8 cells were washed with PBS and resuspended in 1 ml of buffer [10 mM Hepes (pH 7.9)/1.5 mM MgCl_2]. The cells were incubated on ice for 15 min and the nuclei were then pelleted by centrifugation for 15 min at 3000 *g* and 4 °C. The pellet was resuspended in 200 μ l of buffer [30 mM Hepes (pH 7.9)/1.5 mM MgCl_2 /0.2 mM dithiothreitol/1 mM PMSF/1 μ g/ml aprotinin/1 μ g/ml leupeptin/500 mM KCl/10% (v/v) glycerol]. The cells were incubated on ice for 30 min and pelleted by centrifugation for 20 min at 12000 *g* and 4 °C. The supernatant was recovered as the nuclear extract and protein concentrations were determined by a modified Bradford method [23] (Bio-Rad).

For EMSA, 8 μ g of nuclear extract protein was mixed with the binding buffer [30 mM Hepes (pH 7.9)/1.5 mM MgCl_2 /0.2 mM dithiothreitol/1 mM PMSF/1 μ g/ml aprotinin/1 μ g/ml leupeptin/100 mM KCl/10% (v/v) glycerol containing 0.5 μ g of poly(dI-dC)]. Approximately 10000 c.p.m. of double-stranded 32 P-labelled oligonucleotides containing the Sp1 and NE-1 sequences were used as respective probes. In the competition experiments, a 100-fold molar excess of unlabelled oligomers was incubated with the nuclear extracts before the addition of 32 P-labelled oligonucleotides. For supershift analysis, 1 μ l of antibody was incubated with the nuclear extracts at room temperature for 30 min in the binding buffer, followed by an additional incubation for 20 min at room temperature with the labelled probe. Bound and free DNA were resolved by electrophoresis through a 5% (w/v) polyacrylamide gel in $0.25 \times$ TBE for 2 h at 120 V; the gels were dried and exposed for autoradiography overnight at −70 °C.

UV cross-linking of nuclear proteins to DNA

The binding reaction mixture of probe and nuclear extracts was exposed to UV (254 nm) for 60 min at a distance of 2 cm from the light source. The UV cross-linking reaction was blocked by the addition of 5 μ l of 5 $\times$ Laemmli gel loading buffer and subjected to SDS/PAGE [10% (w/v) gel]. One lane was for the prestained protein molecular mass marker (broad range, 7–203 kDa; Bio-Rad). After electrophoresis, the gel was dried under vacuum; the protein directly involved in the DNA interaction was identified by autoradiography.

RESULTS

Cloning of the 5'-flanking region of the mouse dynamin I gene

Screening of a mouse genomic library with a 5' fragment of the mouse dynamin I cDNA identified several bacteriophage clones that contained 5'-flanking regions of this gene. After further characterization by Southern blot analysis, a 6 kb fragment containing 1 kb of the upstream region was subcloned into

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-931                                     T CGATCCAAC
-920 ATCTGTCTGT GTGAAAAGGA CAGGCAAGTA TGGCTGCAAG
-880 AACCCCTGGT TTGAATAGCA CTATTGGCCA CTCTGGGCCT
-840 GCGGCTCATC CTCTGGAGC CTTAGAGTTC AAGACAAGAA
-800 CTTTGCCTC TCCTCGCAG CACTTCCTAG GTATTGGGGC
-760 CTTTGCACCT CTTGTCCACT CCATCAGGGT GTCGGGCCCC
-720 TTCTTATCAG TTAGGTTCCC TTTCAAAGGT AATTTCCTTG
-680 GAAAACCTTT GCCCCTTCTT GGCTAATGTG ACTTTCCAAG
-640 CCCTGTGGT TTTCTCTGTT TCTTCTAGTA CAAACGCTCT
-600 TCTGTGTCCC GTCTATCCCA CCACCTGTAA GATCTCTGAG
-560 TGTAAGTCC CTAGCTGTTT TGCCGAGTTT TGTGCTGTGA
-520 AACTTAGGAA GGGGTCTAGC TTATCATGGG CTCTGTGTAA
-480 TTTGTTGAAT CAATGGTTT TCTTCTTCC TCCTAGAACC
-440 AGAAACACTG AGGCCCCAGG ATAGGAAGGA ATATGCTGCA
-400 GATTTTGTAT TAGTGTGAGA ACCTAGACTA GAAAGGCTAA
-360 GTGGAAGTAG GTTGACCTTG CTCAGCTGAC TGCAGCAGCC
-320 TGTGGGTGCC AGATCTTCCC TCTAAGGCCT CAGTTTCTCC
-280 ACTGGGAATA ACTGCTGAAA TGAAGTAATC ATGACCACCA
      C/EBP $\beta$ 
-240 CATACTAGCC CATTCTAGA GTTAGACCC CTAGCCATCA
-200 CCAAAAGTAT TATCTTCGGT GCAGCATCCG TATCCAGAA
      GATA
-160 ACACAGGTAA GTCTCCCGAA AGCCGCAGAA GAAGAAAACG
      NFAT-1
-120 ACAACACCCA TGCGGCCCGG CGTTGCGTCT GCGCATAGTG
-80 ACCAGTCCGC GAATCCCGCG GCGCATCTA GCGGCGGCCT
      NF- $\kappa$ B [NF-1]
-40 GGAGAAAGCG TCCGCTCGGA TCCCGGAGG GCGGCGGACC
      Sp1
+1 CGCGGCGCAC GCAGTCGGGA TATTCGGCTG CAGCCGCGAA
+41 GCGGGGGGCC AAGAGCCAGA GCAGGAGCGG CGGCCGGATC
+81 GCAGCCCGCC GGGCCCGCCG CAACCATGGG CAACCGCGCG
      AP2 ←EP
+121 ATGGAAGACC TCATCCCGCT GGTAAACCGG TTACAGGACG
+161 CCTTCTCCGC CATCGGCCAG AACGCGGACC TCGACCTGCC
+201 GCAGATCGCC GTGGTAGGCG GCCAGAGCGC CGGCAAGAGC

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Figure 1 Nucleotide sequence of the 5'-flanking region of the dynamin I gene

Shown is the 1173 nt sequence, which contains 931 nt of the 5'-flanking region and 240 nt of the first exon (105 nt of 5' untranslated region and 135 nt of coding sequence). Nucleotide numbering starts at the transcription initiation site (+1), which is indicated by a bent arrow. The first codon (+106 to +108) is shown in bold. Sequences with possible biological significance (AP2, Sp1, C/EBP β , NFAT-1, GATA and NF- κ B) are indicated and are also shown in bold. Boxed regions indicate the critical factor-binding sites for expression of the dynamin I gene, shown in this paper. The binding region of the primer EP used in primer extension analysis is underlined.

Figure 2 Determination of the transcription start site of the dynamin I gene

The transcription start site was mapped by primer extension analysis. A 36-base oligonucleotide, EP, complementary to the coding strand at positions +70 to +105, was end-labelled with [γ - 32 P]ATP by T4 polynucleotide kinase, annealed to 10 μ g of total RNA from NS20Y cells and extended with reverse transcriptase. The extension products were analysed on an 8 M urea/6% (w/v) polyacrylamide gel along with a dideoxy DNA sequencing reaction, with EP as the primer. The lanes G, A, T and C correspond to the nucleotides in the non-coding strand. The 105 nt extension products in lane P are indicated with an arrow. DNA sequences (non-coding strand) surrounding the transcription start site are shown at the left.

pBluescript SK(–) to obtain pBS-dynIP. Figure 1 shows the 1173 nt sequence established from this clone, which contains 931 nt of the 5'-flanking region and 240 nt of the first exon (105 nt of 5' untranslated region and 135 nt of coding sequence). The 5'-flanking sequence was analysed with TRANSFAC [24]. The proximal sequence of the 5'-flanking region is devoid of canonical *cis*-acting elements such as a TATA box or a CAAT box but contains potential binding sites for several transcription factors such as Sp1, NF- κ B and AP2.

Identification of transcription initiation site

Primer extension analysis was performed to determine the transcription initiation site of the mouse dynamin I gene. A 36 nt oligonucleotide (EP) complementary to the coding strand at positions +70 to +105 was used as an extension primer. Total RNA (10 μ g) from mouse NS20Y neuroblastoma cells was hybridized with this probe and extension products were analysed on a 6% (w/v) sequencing gel along with a dideoxy DNA sequencing reaction of pBS-dynIP, with EP as the primer. This yielded a single radiolabelled band 105 nt in length (Figure 2). The nucleotide at this position was designated +1, the start of transcription initiation.

Cell-type-specific expression and functional analysis of the dynamin I gene promoter

To determine whether or not the 5'-flanking sequence of the mouse dynamin I gene contains a cell-type-specific promoter, we constructed a reporter plasmid, pCATIP931, containing a 931 bp 5'-flanking sequence of the dynamin I gene (–931 to +105) fused to the promoterless CAT gene in pCAT-Basic. The construct was assayed for promoter activity by transient trans-

Figure 3 Dynamin I promoter-reporter constructs and their relative CAT activity

(A) Schematic representation of the reporter gene constructs of various lengths for the 5'-flanking region of the dynamin I gene. These constructs were transiently co-transfected with a β -galactosidase expression plasmid into NS20Y (black bars) and lung cells (grey bars). (B) Schematic representation of a series of 5' deletion constructs from the region between -330 and -1. These constructs were transiently co-transfected with a β -galactosidase expression plasmid into NS20Y cells. The promoter activities of each dynamin I-CAT fusion construct were determined and expressed as a multiple of the CAT activity of pCAT-Basic. Results are expressed as means $\pm$ S.E.M. for three independent experiments.

fection into mouse neuroblastoma NS20Y, fibroblast NIH 3T3, Hepa1C1C7 and Lewis lung cells. As a negative control, pCAT-Basic was transfected into parallel cultures of each cell line. The CAT activity from pCATIP931 was normalized to the β -galactosidase activity derived from the co-transfected internal control plasmid pCMV β and shown as multiples of the activity obtained with pCAT-Basic (taken as 1) transfected into that cell line. Mouse neuroblastoma NS20Y cells transiently expressed substantial CAT activity (4-fold), whereas Lewis lung cells showed only minimal CAT activity, not greater than that driven by pCAT-Basic (Figure 3A). Hepa1C1C7 and NIH 3T3 cells expressed an intermediate level of CAT activity (approx. 2-fold; results not shown). These results suggest that the 931 bp 5'-flanking sequence contains sufficient information to direct cell-type-specific expression of the dynamin I gene.

To map the regions that regulate cell-type-specific gene expression, we made a series of reporter plasmids containing progressive 5' deletions between nt -931 and 0. Because the construct pCATIP931 expressed substantial CAT activity in NS20Y cells but not in lung cells, these chimaeric constructs were transfected into these two cell lines (Figure 3A). Lewis lung cells showed only minimal CAT activity for all deletion constructs. In NS20Y cells, a slight decrease in promoter activity was detected as the 5'-deletion mutation approached -731 relative to the transcription start site. Further deletion from -730 to -531 increased the promoter activity, suggesting the presence of a negative element in this region. Deletion up to nt -331 had little effect on promoter activity. Extension of the 5' deletion to nt -1 drastically decreased the promoter activity to that seen with the promoterless control vector. These results demonstrate that the

Figure 4 Effects of mutations in the NE-1 and Sp1 elements on mouse dynamin I gene promoter activity

(A) Left panel: schematic representation of the reporter gene constructs pCATIP90, pCATIP90mNE-1 and pCATIP60. Right panel: CAT activity observed in NS20Y cells transfected with these reporter gene constructs. (B) Left panel: schematic representation of the reporter gene constructs pCATIP30, pCATIP30mSp1 and pCATIP0. Right panel: CAT activity observed in NS20Y transfected with these reporter gene constructs. Results are expressed as means $\pm$ S.E.M. for three independent experiments.

sequence between -330 and -1 is necessary for cell-type-specific promoter activity.

To identify the regulatory region more precisely, a series of 5' deletion constructs from between -330 and -1 were transfected into NS20Y cells (Figure 3B). Sequential deletion of the region between nt -330 and -120 had little effect on CAT activity. Further deletion from -120 to -90 increased the promoter activity, suggesting the presence of a negative element in this region. Further analysis of the dynamin I promoter revealed that construct pCATIP90, containing a fragment from -90 to +105, showed the highest promoter activity. Deletion from -90 to -61 (pCATIP60) led to a significant decrease in promoter activity compared with pCATIP90. Construct pCATIP30 has a substantial promoter activity compared with pCATIP0. These findings suggest that cell-type-specific and constitutive promoter activities of the dynamin I gene are dependent on elements spanning -90 to -61 and -30 to -1.

Contribution of the Sp1-binding site and NE-1 to the promoter activity of the mouse dynamin I gene

Because plasmid pCATIP90 was the shortest construct with the highest promoter activity, we used this construct to define further the regulatory element on the dynamin I promoter activity. A Sp1-binding site and NE-1 were present in this region (-90 to -1). To define whether these two elements contribute to the dynamin I promoter activity, the Sp1-binding site and NE-1 sequences were mutated and the effects of each mutation on promoter activity were determined in NS20Y cells. Mutation of the NE-1 sequence in pCATIP90 decreased promoter activity to the lower level of the truncated promoter construct pCATIP60, in which the 30 bp upstream of pCATIP90, containing NE-1, was removed (Figure 4A). The mutation of the Sp1 element also decreased promoter activity significantly (Figure 4B). These results suggest that Sp1 and NE-1 are required for display of the proper expression levels of the dynamin I gene in neuronal cells.

Figure 5 EMSA with Sp1 element of the mouse dynamin I promoter

EMSA was performed with 8 μ g of NS20Y nuclear extract and labelled Sp1 probe. A specific DNA–protein complex was formed when the labelled Sp1 oligonucleotide was used (lane 1). Competition analysis was performed in the presence of a 100-fold molar excess of unlabelled Sp1 (lane 2), mSp1 (lane 3) and NF- κ B (lane 4) oligonucleotide. Supershift analysis was performed in the presence of anti-p65 (lane 5) and anti-Sp1 (lane 6) antibody (Ab).

Transcription factor Sp1 binds to the Sp1 element

The construct pCATIP30 has a substantial promoter activity compared with pCATIP0; the Sp1 element in this region is critical for promoter activity of the dynamin I gene (Figures 3 and 4). To determine the DNA–protein complexes forming over this region, we performed an EMSA analysis. The oligonucleotide spanning –30 to –11 did not form any specific DNA–protein complex (results not shown). However, a specific DNA–protein complex was formed when the oligonucleotide spanning –20 to –1 was used as a probe (Figure 5, lane 1). Complex formation was inhibited specifically by adding excess amounts of unlabelled Sp1 consensus oligonucleotide (Figure 5, lane 2) but not by the Sp1 mutant oligonucleotide (lane 3), indicating that these DNA–protein complexes were formed by Sp1-specific binding protein and the Sp1 oligonucleotide. Indeed, in a subsequent supershift analysis with a mouse Sp1-specific monoclonal antibody, the addition of antibody further decreased the mobility of the majority of these DNA–protein complexes (Figure 5, lane 6). Taken together, the results further demonstrate directly that Sp1 binds to the Sp1 element spanning –13 to –4.

Factor binding the NF- κ B-like element

NE-1, spanning –73 to –64, is crucial for the promoter activity of the dynamin I gene (Figure 4). We therefore performed an EMSA analysis to determine whether or not NF- κ B binds to this element. A specific DNA–protein complex was formed when the oligonucleotide spanning –75 to –56 (NE-1) was used as a probe (Figure 6A, lane 1). Complex formation was inhibited specifically by adding excess unlabelled NE-1 oligonucleotide (Figure 6A, lane 2). Surprisingly, however, the NF- κ B consensus

Figure 6 EMSA analysis with the NE-1 region of the mouse dynamin I promoter

(A) EMSA was performed with 8 μ g of NS20Y nuclear extract and labelled NE-1 probe. A specific DNA–protein complex was formed when the labelled NE-1 oligonucleotide was used (lane 1). Competition analysis was performed in the presence of a 100-fold molar excess of unlabelled NE-1 (lane 2) and NF- κ B (lane 3) oligonucleotide. (B) Supershift analysis was performed in the presence of anti-p50 (lane 2), anti-p65 (lane 3), anti-RelB (lane 4) and anti-c-Rel (lane 5) antibody (Ab). (C) EMSA was performed with 8 μ g of nuclear extracts of mouse pre-B lymphocyte 70Z/3 cells, activated by 1 μ g/ml of lipopolysaccharide for 2 h, with labelled NF- κ B probe. Specific DNA–protein complexes were formed (lane 1, arrows). Supershift analysis was performed in the presence of anti-p65 (lane 2), anti-p50 (lane 3), anti-RelB (lane 4) and anti-c-Rel (lane 5) antibody. The supershifted complex in lanes 2 and 3 is indicated by an arrowhead.

oligonucleotide did not inhibit the formation of the specific DNA–protein complex (Figure 6A, lane 3). We also tested subsequent supershift analyses with NF- κ B-specific monoclonal or polyclonal antibodies (p50, p65, RelB and c-Rel) but antibody addition did not alter the mobility of these DNA–protein complexes (Figure 6B). To confirm that the antibodies that we used interacted with NF- κ B if it was present in nuclear extracts, we performed EMSA with nuclear extracts of mouse pre-B lymphoma 70Z/3 cells that had been treated with 1 μ g/ml lipopolysaccharide for 2 h using the NF- κ B probe (Figure 6C). Two specific complexes (Figure 6C, lane 1, arrows) were formed; we then performed supershift analyses to examine which subunit of NF- κ B was contained in these complexes. A supershifted complex was formed when p65 and p50 antibodies were added to the reaction (Figure 6C, lane 2 and 3 respectively, arrowhead). It has been shown that the transcription factor NF- κ B in 70Z/3 cells is activated by treatment with lipopolysaccharide [25]. These results indicate that NEBP is not NF- κ B.

Next, to delineate the core-binding site for NEBP in NE-1, we performed EMSA competition analysis with a series of mutant oligonucleotides (m1NE-1 to m5NE-1). DNA–protein complex formation was specifically inhibited by the addition of excess unlabelled m4NE-1 and m5NE-1 oligonucleotides (Figure 7A, lanes 6 and 7) but not by the oligonucleotides m2NE-1 and m3NE-1 (lanes 4 and 5). These results suggest that CGAATCCC sequences in NE-1 are critical for the binding of NEBP to NE-1. Because oligonucleotide m1NE-1 did not inhibit the specific complex formation completely (Figure 7A, lane 3), we conclude

Figure 7 Determination of the core sequence in NE-1 region essential for NEBP binding

EMSA was performed as described in the legend to Figure 6. (A) A 100-fold excess of wild-type or mutant NE-1 oligonucleotide (m1NE-1 to m5NE-1) was used as unlabelled competitor. (B) Wild-type or mutant ER-1 oligonucleotide (m1, m2 and m12NEkB) was used as unlabelled competitor. (C) Oligonucleotides used in these experiments.

that the TCCG sequence in the NE-1 region partly contributes to complex formation.

As shown in Figure 7(C), the NE-1 core sequence differs in a few nucleotides from a consensus NF-κB-binding site. However, the NF-κB consensus oligonucleotide did not inhibit the formation of a specific DNA-protein complex (Figure 6A, lane 3). To assess whether these subtle sequence differences have any role in binding specificity to discriminate between NEBP and NF-κB, we synthesized mutant NE-1 oligonucleotides containing one or more mutations in the NE-1 core sequence GGGATTTCGCGGA, and performed EMSA competition analysis (Figure 7B). The mutation of the underlined G residue within the NE-1 core sequence GGGATTTCGCGGA to C did not inhibit DNA-protein complex formation (Figure 7B, lane 3) but altering the next G to C completely inhibited specific complex formation (lane 4). The oligonucleotide with double mutations had no inhibitory effect (Figure 7B, lane 5). These results demonstrate that one base in the NE-1 core sequence is critical for binding specificity to discriminate between NEBP and NF-κB.

UV cross-linking of the NE-1-binding complex

To determine the molecular mass of the activator component directly in contact with the NE-1 and to estimate the numbers of

Figure 8 UV cross-linking of NS20Y nuclear proteins to the NE-1 motif

Crude nuclear NS20Y extracts were incubated with radiolabelled NE-1 probe in the absence (lane 1) or presence (lane 2) of unlabelled competitor, followed by exposure to UV and separation by SDS/PAGE. The position of the UV cross-linked protein band is indicated by an arrow. The positions of molecular-mass markers are indicated (in kDa) at the right.

molecules involved, we performed UV cross-linking of NS20Y nuclear extracts to the labelled NE-1 probe. For this, radiolabelled DNA-protein complex was exposed to UV (254 nm) for 60 min before gel separation by SDS/PAGE [10% (w/v) gel]. As shown in Figure 8, a single band of approx. 124 kDa comprising the DNA-protein complex was identified (lane 1). Complex formation was specifically inhibited by adding excess unlabelled NE-1 oligonucleotide to the binding reaction (Figure 8, lane 2). By subtracting the molecular mass of the NE-1 probe, the molecular mass of NEBP was estimated as approx. 104 kDa. Similar results were obtained in a repeat experiment.

DISCUSSION

In brain, three different isoforms of dynamin (brain-specific dynamin I, ubiquitously expressed dynamin II, and dynamin III, which was originally described as being testis-specific) are expressed simultaneously. The mRNA levels of dynamin I and III are also up-regulated throughout brain development, whereas the levels of dynamin II mRNA remain unchanged. Therefore, for an understanding of the role of this protein *in vivo*, it is important to establish the regulation mechanism that confers cell-type and development-stage specificity of dynamin I. For this purpose we performed molecular cloning and functional characterization of the upstream regulatory region of the mouse dynamin I gene. On the basis of the results of 5'-end serial deletion analyses, we localized the core promoter controlling this expression to a minimal region situated between -90 and -1 bp upstream of the transcription initiation site of dynamin I.

From our results, two *cis*-elements were identified as being crucial for the dynamin I promoter activity. The first of these is the Sp1 element. The transcription factor Sp1 was shown to bind this element (Figure 5). Sp1 is a well-characterized sequence-specific DNA-binding protein that is important in the transcription of many cellular and viral genes that contain GC boxes in their promoters [26-30]. In all of these promoters, Sp1 elements are required for efficient transcription. For many TATA-less promoters, activation requires the multisubunit TFIID complex [31,32], with Sp1 binding at GC boxes functioning to stabilize the interaction of TFIID with the transcription start site [33,34]. We demonstrated that Sp1 is critical for the dynamin I gene expression by showing that Sp1 was the major binding factor on

the Sp1 element and that mutation of the Sp1 element resulted in an decrease in the promoter activity in NS20Y cells.

The NF- κ B-like element, named NE-1 in this study, is another crucial *cis*-element for dynamin I promoter activity. NE-1 includes a potential binding site for the transcription factor NF- κ B but we showed that the major protein (NEBP) that binds to the NE-1 element is not NF- κ B (Figure 6). However, NEBP has a binding sequence that is very similar to the consensus sequence of the NF- κ B-binding site. We also defined that the underlined G residue within the GGGATTCGCGGA motif is critical for binding specificity to discriminate between NEBP and NF- κ B. Several factors other than NF- κ B have been shown to interact with NF- κ B-like motifs, including AP-3, MP-1, PRDII-BF1, HIV-EP1 and MBP1 [35–39]. Briegel et al. [40] showed that TCF-1 binds more strongly than NF- κ B to an NF- κ B-like motif (TCed) in the interleukin 2 promoter. They demonstrated that the conversion of TCed to a perfect NF- κ B-binding site leads to a tighter binding of NF- κ B to TCed DNA and abolishes its T-cell-restricted activity. They also proved that a change in one nucleotide determines whether this NF- κ B-like proto-enhancer motif acts as a cell-type-specific or an ubiquitous enhancer motif. In our study we demonstrated that NE-1 is a critical element of dynamin I gene expression by showing that a transcription factor (NEBP) was able to bind to the NE-1 element and that mutation of the NE-1 sequence resulted in a decrease in the promoter activity in NS20Y cells.

As mentioned above, dynamin I gene expression is high in brain and its expression is regulated during brain development. The 5'-promoter region of the dynamin I gene displays a strong activity in the neuronal cell line. However, Sp1 and NEBP, which are themselves critical for dynamin I promoter activity, are not neuron-specific transcription factors (NEBP formed a specific DNA-protein complex with NE-1 in lung cell nuclear extracts; results not shown). However, the promoter activity of dynamin I, which seems to be strongly dependent on the ubiquitously expressed Sp1 and NEBP, might be regulated in a tissue-specific manner by these factors in several ways. The first possibility is the availability of these factors; Saffer et al. [41] showed that the levels of Sp1 protein vary between different tissues. They showed that Sp1 expression varies greatly in different cell types and that changes in Sp1 levels occur during development. From these results they suggested that, although Sp1 is ubiquitously expressed, substantial variations that are likely to have an important regulatory role occur in Sp1 expression. Another possibility is that binding of some cell-type-specific transcription factors, which allow cell-type-specific activity of the dynamin I promoter, to its binding site is dependent on the interaction with Sp1 or NEBP; Tretiakova et al. [42] showed that binding of Puralpha, a cell-type-specific transcription factor, to its binding site, is enhanced by inclusion of Sp1 in the binding reaction. They also showed that transient interaction of Puralpha and Sp1 might result in a stable association of Puralpha with its binding element and these factors synergistically stimulate myelin-basic-protein promoter activity in cells of the central nervous system. Further studies involving identification of proteins that bind with Sp1 or NEBP will therefore facilitate the elucidation of the tissue-specific and developmental-stage-specific expression of the dynamin I gene. We also tried to find the negative element in the region between -120 to -90, and found that YY-1 binding element is present in this region (J. Yoo and M. Y. Han, unpublished work).

In summary, we have demonstrated for the first time the importance of elements within the proximal region of the mouse dynamin I promoter for cell-type-specific expression. This analysis also provides novel information on how the dynamin I gene

is regulated in mouse neuroblastoma cells. Given the importance of the Sp1 and NE-1 sequences for cell-type-specific expression, it will be of interest to characterize NEBP and find other transcription factors that interact with Sp1 or NEBP.

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