Isolation and Characterization of a Novel Member of the Neural Zinc Finger Factor/Myelin Transcription Factor Family with Transcriptional Repression Activity*

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Myelin transcription factor 1 (MyT1) and neural zinc factor 1 (NZF-1) represent the first two members of an emerging family of neural specific, zinc finger-containing DNA-binding proteins. MyT1 has been shown recently to play a critical role in neuronal cell differentiation during development. We have cloned the third member of the NZF/MyT family, referred to as neural zinc finger factor 3 (NZF-3). The cDNA sequence predicts a protein of 1,032 amino acids which contains two clusters of zinc fingers similar to MyT1 and NZF-1. Unlike MyT1 and NZF-1, NZF-3 does not contain an acidic domain at the amino terminus or a serine/threonine-rich region between the two finger clusters. NZF-3 binds to a DNA element containing a single copy of the previously described AAAGTTT consensus motif for these factors but exhibits a marked enhancement in relative affinity to a bipartite element containing two copies of the consensus motif. In contrast to MyT1 and NZF-1, which are known to activate transcription, cotransfection experiments revealed that NZF-3 confers repression on the basal activity of promoters containing the consensus binding elements. The identification of an additional member of the NZF/MyT family provides an opportunity to investigate the relative contribution of members of this family of transcription factors to the complex regulatory processes in neural development and homeostasis.

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The role of cell type-specific transcription factors in lineage specification during invertebrate development (1), vertebrate development (2), vertebrate myogenesis (3), and development of the immune system (4, 5) is well established. In each of these cases, critical decisions concerning cell fate involve transcription factors that are expressed in the earliest precursors of a particular cell type. If this paradigm also holds true in the generation of specific neuronal lineages in the developing nervous system, the identification of transcription factors marking neuronal cell types from their birth in the neural tubes and throughout their complex program of differentiation is a critical step in advancing our understanding of these cell fate decisions.

Zinc-coordinated fingers are one of the most common DNA binding motifs among eukaryotic transcription factors and are classified according to the number and position of the cysteine and histidine residues available for zinc coordination. The Cys-His-His (C2H2) class, which is typified by the Xenopus transcription factor IIIA (6), contains the largest number of members. These proteins contain two or more fingers arranged in tandem. In contrast, steroid receptors contain only two zinc-coordinated structures with four (C4) and five (C5) conserved cysteines. The third class of zinc fingers, which binds to single-stranded nucleic acids, has a consensus sequence of Cys-X2-Cys-X7-His-X4-Cys (C4HC). Such factors are found in mammals (7), Drosophila transposable element copia (8), and in retroviruses (9). Other metal-coordinating proteins have different structures such as C6 in the yeast GAL4 protein and a cysteine-rich structure in the E1A oncoprotein (10). In accordance with their structural diversity, zinc finger proteins play a variety of important roles in cell growth, differentiation, and development. Transcription factor IIIA and the ubiquitous transcription factor SP1 are broadly involved in the regulation of transcription, whereas the Drosophila zinc finger proteins Krüppel and Hunchback are crucial for proper segmentation of the developing embryo (11–13). The zinc finger protein REST (RE1-silencing transcription factor) has been shown to repress neuronal gene expression in non-neuronal tissues (14, 15).

Two zinc finger proteins, neural specific zinc finger factor 1 (NZF-1) (16) and myelin transcription factor 1 (MyT1, also known as NZF-2) (17), have recently been identified by virtue of their ability to bind the cis-regulatory element present in the promoter of the β-retinoic acid receptor and myelin proteolipid protein genes, respectively. Each of these proteins contains six Cys-X5-Cys-X12-His-X4-Cys (C4HC) type zinc fingers of novel configuration, different from the previously described C4HC type of zinc fingers found in proteins present in retroviruses (9). The zinc fingers in these proteins are arranged in two main clusters. Each of these clusters can independently bind DNA and recognize similar core consensus sequences. Other motifs that can be found in these proteins include an acidic domain at the amino terminus and a serine/threonine-rich domain situated in the region between the two zinc finger clusters. These proteins are highly homologous to each other, suggesting that...

1 The abbreviations used are: NZF-1, neural specific zinc finger 1; MyT1, myelin transcription factor 1 (also known as NZF-2); PCR, polymerase chain reaction; NZF-3, neural zinc finger 3; kb, kilobase pair(s); GST, glutathione S-transferase; PBS, phosphate-buffered saline; CMV, cytomegalovirus; EMSA, electrophoretic mobility shift assay.
they belong to a subfamily of zinc-finger-containing transcription factors. In addition, Northern analysis revealed that they are highly enriched in the brain (16, 17). The restricted pattern of expression of these proteins indicates that they may serve important functions in the development and maintenance of the nervous system. Indeed, in the course of this study, an independent report demonstrated that X-MyT1, a Xenopus homolog of MyT1, serves an important function in the primary selection of neuronal precursor cells in the developing embryo (18). X-MyT1 is able to promote ectopic neuronal differentiation in cooperation with the basic helix-loop-helix neural transcription factors (18). Furthermore, normal neurogenesis and ectopic neurogenesis caused by overexpression of the neural basic helix-loop-helix factors are inhibited by a dominant negative form of X-MyT1 (18).

Given the high possibility of members of the NZF/MyT family being involved in neural development and homeostasis, it would be valuable to identify and characterize novel members of this family. Thus, a strategy was devised to isolate new members of the NZF/MyT gene family. Degenerate oligonucleotides representing all possible codons for two stretches of 11 amino acid residues conserved between NZF-1 and MyT1 were used as primers in the polymerase chain reaction (PCR). DNA complementary to poly(A)-selected RNA from human brain, thymus, liver, spleen, kidney, placenta, and rat brain were used as templates. Here, we describe the cloning and characterization of a novel member of the NZF/MyT family, referred to as neural zinc finger factor 3 (NZF-3).

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning and Isolation**—Degenerate primers were designed based on two conserved regions (non-zinc finger) between human MyT1 and rat NZF-1 (Fig. 1A). The amino acid sequence and degenerate nucleotide sequence of the 3′-primer were EILAMHENLVR and 5′-GA/G/A/AT/CT/T/CT/TT/T/A/GC/CCATGCTAGA/G/GAA/AC/CT/GT-AT/G/C/CT/G/CG/GAAG-3′, respectively. Those of the 3′-primer were EV-DENGLTLDS and 5′-GCT/AG/AG/G/GTC/GA/A/G/GA/AC/GG/GA/CG/GA/CT/GA/AA/G/G/CG/CT/GA/GG/GA/CG/GA/GG/GA/AG-3′ respectively.

cDNA from the following tissues was used as a template in the PCR: human thymus, thymus, liver, spleen, kidney, placenta (all purchased from CLONTECH, Palo Alto, CA) and rat brain (CLONTECH). 40 PCR cycles were performed: denaturation at 94 °C for 30 s, annealing at 42 °C for 2 min, and extension at 72 °C for 30 s. PCR products were amplified from human and rat brain cDNA. The ends of the PCR products were repaired with the Klenow fragment of DNA polymerase I (New England Biolabs), phosphorylated with T4 polynucleotide kinase (New England Biolabs), and subcloned into the pBluescript II (Stratagene, La Jolla, CA) according to standard procedures (19). These products were subsequently sequenced.

A random primed 32P-labeled probe was prepared from a novel fragment amplified from rat brain cDNA and used to screen a rat brain Agt11 cDNA library by DNA hybridization. Phages were plated, transferred to nitrocellulose filters, and densitometrized on a standard procedure (19). After being baked, the filters were prehybridized for 2 h at 55 °C. A solution composed of 0.5 mM sodium phosphate buffer (pH 7.0), 7% SDS, 15% formamide, and 0.5% SDS several times at room temperature for 30 min followed by washes in 0.1 × SSC and 0.2% SDS at 65 °C for another 30 min before autoradiography. The blot was later stripped and rehybridized with a β-actin probe.

Total RNA was also prepared from selected rat tissues and cell lines using Ultraspec™ RNA solution (Biotec Inc., Houston) and used in ribonuclease protection assays (Ambion) according to the manufacturer’s instructions. Hybridization reactions contained 25 μg of total RNA. Antisense ribonucleotide probes were transcribed in vitro in the presence of [α-32P]dCTP from a pBKS construct containing a 351-base pair SacI-StuI fragment (nucleotides 1711–2062) and were gel purified. This fragment corresponded to a portion of the region between the two zinc finger clusters of NZF-3. RNA-resistant fragments were resolved by electrophoresis on an 8% urea, 6% polyacrylamide gel.

**Protein Expression and Antibodies**—Fragments of NZF-3 cDNA were subcloned into the pGEX-KG bacterial expression vector (20) to produce glutathione S-transferase (GST) fusion proteins. The GST-2ZF construct consisting of the first two fingers of the four-zinc finger cluster with additional upstream sequences (amino acids 601–796) was generated using a PCR-based strategy. The GST-4ZF construct, which contains the entire four-zinc finger cluster, was generated by subcloning a 0.8-kb SacI-StuI (coding for amino acids 668–962) fragment into the EcoRV and Accl sites of pBKSII(−) (21). A fragment was then excised using EcoRI and Xhol and cloned into the corresponding sites in pGEX-KG. The recombinant plasmids or vector alone was introduced into the Escherichia coli BL21 (DE3) strain (21). Transformed bacteria were grown to an A600 of 0.8 and then induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. After 3 h the bacterial cells were harvested and centrifuged.

Bacterial pellets containing insoluble GST-2ZF fusion protein were resuspended in SDS sample buffer (2% SDS, 100 mM Tris (pH 7.5), 280 mM β-mercaptoethanol, and 20% glycerol). After heating and samples centrifugation, the supernatant was electrophoresed in preparative SDS-polyacrylamide gels. The expressed GST-2ZF protein was visualized with Coomassie Blue and excised from the gel. Subsequently, the protein was eluted from the gel and used to immunize rabbits to raise antibodies. Bacterial pellets containing the GST-4ZF fusion protein were resuspended in lysis buffer (0.4 mM NaCl, 50 mM Tris (pH 7.6), 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin) and sonicated following centrifugation. The supernatant fraction containing the bacterial extract was analyzed using SDS-polyacrylamide gel electrophoresis and Western blot. This extract was used in DNA binding experiments.

**Immunoblot Analysis**—Protein samples (bacterial and mammalian cell extracts) were mixed with SDS sample buffer, fractionated on a 10% SDS-polyacrylamide gel, and then transferred to a 0.2-mm pore size nitrocellulose membrane (Amersham). Membranes were blocked in phosphate-buffered saline (PBS) and then incubated with rabbit polyclonal antibody (preimmune and anti-GST-2ZF immune sera) diluted 1:10,000 in 5% non-fat milk for 1 h before incubation with rabbit polyclonal antibody (preimmune and anti-GST-2ZF immune sera) diluted 1:10,000 in PBS-T containing 1% non-fat milk. The blots were washed three times with PBS-T, incubated with a 1:15,000 dilution of horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham) for 1 h, washed three times, and visualized using the Enhanced Chemiluminescence (ECL) system (Amersham).

**Whole Cell, Nuclear, and Cytoplasmic Extract Preparation**—Full-length NZF-3 cDNA was cloned into the BamHI-XhoI sites of the pXJ40 mammalian expression vector, which is under the control of the cyto-megalovirus (CMV) promoter/enhancer (22). Transfections were carried out in 293 human embryonic kidney cells that were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Hyclone). The cells were transfected with either pXJ40 or pXJ40NZF-3 using Lipofectin reagent (Life Technologies, Inc.) and harvested 24 h later. Whole cell extracts were prepared by resuspending the transfected cells in binding buffer used for electrophoretic mobility shift assays (EMSAs), freezing them at −80 °C, thawing them over ice, and centrifuging at full speed for 15 min at 4 °C. The supernatant was used in EMSAs. Nuclear extracts were prepared on double sucrose (23) except that the cytoplasmic fraction was retained for analysis. The fractions were separated by SDS-polyacrylamide gel electrophoresis and subjected to Western blot analysis.

**Immunofluorescence Staining**—293 cells were seeded onto glass coverslips and transfected with either pXJ40 or pXJ40-NZF-3 expression plasmid. 24 h after transfection, the cells were washed in ice-cold PBS,
**FIG. 1. Cloning of NZF-3.** Panel A, cloning strategy for identification of a novel member of the NZF/MyT family. On the schematic diagram of NZF-1 and hMyT1 the positions of zinc fingers are indicated by hatched boxes. Amino acid sequences chosen for designing degenerate primers for **PCR** are as indicated. Panel B, cloning of a full-length cDNA of NZF-3. Full-length cDNA encoding NZF-3 was assembled from three overlapping clones (K1, K2, and K3) obtained from screening a *lambda*11 rat brain cDNA library. Panel C, primary DNA and deduced amino acid sequences of NZF-3. The six Cys-Cys, His-Cys zinc fingers are boxed. Arrows indicate the conserved amino acid region corresponding to the primers used in PCR. The polyadenylation signal at the 3'-end is underlined.
fixed in ice-cold methanol for 5 min at -20 °C, washed in PBS, and incubated in blocking buffer (5% goat serum, 5% fetal calf serum, 2% bovine serum albumin in PBS) for 1 h. The cells were then incubated with primary antibody (preimmune sera and anti-GST-ZF2F immune sera) diluted 1:150 in blocking buffer for 1 h, washed three times with PBS, and then incubated with the fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (Molecular Probes Inc., Eugene, OR) for another hour. After three washes in PBS for 10 min each, the coverslips were mounted in 80% glycerol in PBS containing 1 mg/ml p-phenylene diamine and examined with a Zeiss Axioplan microscope.

EMSA—Complementary oligonucleotides for the SIN element, containing a single copy of the AAAGTTT core motif, were synthesized to carry out analysis on the DNA binding properties of NZF-3. In addition, a series of complementary oligonucleotides based on a direct repeat of the AAAGTTT core motif was synthesized. The spacing between the repeats in the oligonucleotides DR2, DR5, DR7, DR9, and DR11 were 2, 5, 7, 9, and 11 nucleotides, respectively. The BamH I site at the 5'-end and a BglII site at the 3'-end were added for cloning purposes.

The sequences of oligonucleotides used in EMSA are as follows:

**SIN:**

5'-gatccGGATCCCAAGTTTGCATATCA  
5'-gatctATATCAAGTTTGCATATCA

**DR2:**

5'-gatccGGAAAGTTTGCAAGTTTGA  
5'-gatctCAAATGTTTCGCAGTTTGA

**DR5:**

5'-gatccGGAAAGTTTGCCGAAAGTTTGA  
5'-gatctCAAATGTTTCGCAGTTTGA

**DR7:**

5'-gatccGGAAAGTTTGCGTATCAGTTTGA  
5'-gatctCTAAATGCAGCTAGTTTGA

**DR9:**

5'-gatccGGAAAGTTTGCGCGAAAGTTTGA  
5'-gatctCTAAATGCAGCTAGTTTGA

**DR11:**

5'-gatccGGAAAGTTTGCGCGCGAAAGTTTGA  
5'-gatctCTAAATGCAGCTAGTTTGA

**EMSA OLIGONUCLEOTIDES**

Bacterial extracts containing GST-4ZF fusion protein and whole cell extracts from transfected mammalian cells were used in EMSAs as described previously (24).

Competition and supershift reactions were performed by preincubating the reaction mixture with unlabeled oligonucleotide or immune sera for 15 min at room temperature before addition of the radiolabeled probe. Quantitative competitive EMSAs were used to compare relative affinities of different DNA-binding elements for NZF-3. In these experiments, the DR5 element was end labeled and used as a probe. Increasing amounts of unlabeled competitor were added to the sample 15 min before the addition of the radiolabeled DR5 probe. After electrophoresis, the quantity of shifted probe of each sample was determined using a PhosphorImager (Molecular Dynamics). The amount of shifted probe in the absence of competitor was defined as the amount of maximal binding. The fraction of maximal binding at each competitor concentration was then calculated as the ratio of bound probe in the presence of competitor to that in the absence of competitor. To assess the effect of the competitors visually, the fraction of maximal binding was plotted against the amount of unlabeled competitor used. This allowed us to estimate the IC	extsubscript{50} or the concentration of competitor which inhibited maximal binding by 50%. The IC	extsubscript{50} was taken as an indication of the relative binding affinity of a particular oligonucleotide for NZF-3.

**Mammalian Cell Transfection—Oligonucleotides containing single or double (bipartite) copies of the consensus motif (described earlier) were inserted upstream of a minimal herpes simplex virus thymidine kinase promoter driving a luciferase reporter gene (designated Tk-luc) to generate reporter plasmids. In addition, reporter plasmids containing unrelated DNA sequences were also generated. The sequences are as follows:**

5'-gatccCTCGGGACTCGGTCGCTTGAGTA  
5'-gatccCCGCGAGTGTCGCTTGAGTA

**53 CONSENSUS BINDING SITE**

5'-gatccTTGGCCCTCTGGAGGGAAGGAGGAGGACAGGAGA  
5'-gatccGGTGCTGCTCTGGCACCTCACCGAGGACAGAGA

**THYROID HORMONE RESPONSE ELEMENT**

Deletion mutants of NZF-3 were generated using a PCR-based protocol and cloned into the BamHI and KpnI sites of the pXJ40 mammalian expression vector.

All transfections were carried out in 293 human embryonic kidney cells, which were plated out 24 h before transfection. In the deletion mutant analysis, 1.5 μg of reporter plasmid and 1 μg of the pXJ40 vector, pXJ40 NZF-3, or deletion mutant expression plasmids was used in transient transfections using Lipofectin. In addition, 0.2 μg of a β-galactosidase expression vector, pCMV-β-Gal, was also cotransfected in each dish. 48 h after transfection, the cells were rinsed with PBS and lysed in the following buffer: 50 mM potassium phosphate (pH 7.8), 1 mM dithiothreitol, and 1% Triton X-100. The lysate was cleared of cellular debris by centrifugation and analyzed using a Monolight 2010 Luminometer (Analytical Luminescence Laboratories, Ann Arbor, MI). Experiments were performed at least three times in duplicate, and results were normalized with β-galactosidase activity to correct for transfection efficiency.

**RESULTS**

**Amplification of cDNA for NZF/MyT Proteins**—Degenerate primers corresponding to two stretches of conserved sequences in MyT1 and NZF-1 (Fig. 1A) were used in PCR. Because it is unclear whether novel members of this family are expressed in tissues other than brain, cDNAs from several other tissue sources, including kidney, liver, spleen, thymus, and placenta, were also used. PCR products were cloned and sequenced. NZF-1 and MyT1 cDNA fragments were amplified readily from samples derived from both human and rat brain but not from any other tissues. In addition, a novel cDNA clone containing an open reading frame encoding a polypeptide that showed substantial homology to the zinc finger as well as the inter-zinc finger region of NZF-1 and MyT1 were isolated from the brain. The encoded protein thus represents a putative novel member of the NZF/MyT family and is referred to as NZF-3.

**Cloning of a Full-length cDNA of NZF-3**—The rat whole brain cDNA library was probed with a mixture of random primed radiolabeled fragments from the unique PCR fragment encoding the putative novel member. Multiple clones were obtained and analyzed by restriction mapping and in several cases, by partial sequence determination. Three of the overlapping clones, spanning a region of approximately 3.5 kb, were sequenced completely on both strands (Fig. 1B). The assembled cDNA sequence contains a single open reading frame encoding a protein of 1,032 amino acids (Fig. 1C). The 5'-region of the assembled cDNA clone contains stop codons in all three reading frames preceding the predicted translation start site. The 3'-untranslated region encompasses a putative polyadenylation signal (Fig. 1C). Analysis of the protein sequence of NZF-3 revealed that it contains six zinc fingers of the Cys-X<sub>2</sub>-Cys-X<sub>2</sub>-His-X<sub>2</sub>-Cys type. The fingers are arranged in two clusters of two and four, most similar to MyT1 (Fig. 2A). The amino acid sequence of the zinc fingers among members of this family displays a very high degree of conservation, suggesting that they may recognize similar cis-regulatory elements (Fig. 2B).

In addition to the zinc finger domain, the carboxy terminus of NZF-3 also displays a high degree of homology to MyT1 and NZF-1 (Fig. 2A). However, in contrast to MyT1 and NZF-1, NZF-3 does not contain acidic domain at the amino terminus or a serine/threonine-rich motif in the region between the two finger clusters (Fig. 2A).

**Expression of NZF-3 mRNA**—Northern blot analysis was performed to determine whether NZF-3 has a restricted pattern of expression. We screened a rat multiple tissue Northern blot and observed that a NZF-3 transcript of 7.5 kb was expressed in the brain and was completely absent in the heart, spleen, liver, muscle, and kidney (Fig. 3A). A smaller and less abundant transcript was also detected in the testis. This may represent an alternatively spliced form of NZF-3 or other homologous transcripts. RNase protection assay utilizing a specific probe corresponding to the inter-zinc finger clusters region of the cDNA confirmed the results from the Northern analysis that the NZF-3 message was highly restricted to the brain (Fig. 3A).
Subcellular Localization of NZF-3—Polyclonal antibodies raised against the bacterially expressed GST fusion proteins containing the first two fingers (GST-2ZF) of the four-finger cluster of NZF-3 were used to determine the subcellular localization of NZF-3. The antibody was shown to be specific as it recognized the GST-2ZF fusion protein in Western blot analysis but not the GST or any other unrelated proteins in the bacterial extracts (Fig. 4A). The antibody, however, failed to detect the endogenous NZF-3 in PC12 cells by Western immunoblot as well as immunohistochemical analysis (data not shown). The inability of the antibody to detect NZF-3 protein in PC12 cells may be attributed to a low level of expression of the protein in these cells. We therefore decided to overexpress the NZF-3 protein by transient transfection in mammalian cells. 293 human embryonic kidney cells, devoid of any NZF-3 mRNA as determined by RNase protection experiments, were transfected with an expression plasmid containing the full-length NZF-3 cDNA. Cells were incubated with anti-GST-2ZF immune serum followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG and visualized using fluorescence microscopy.
copies of an AAA(G/C)TTT motif arranged in tandem with a variable spacing from 1 to 11 nucleotides (18).

To characterize the in vitro binding properties of NZF-3, whole cell extracts of 293 cells transiently expressing NZF-3 were tested in EMSAs with the DR5 element. Unlabeled competitor oligonucleotides and antisera were added as indicated. Two DNA-protein complexes, C1 and C2, were detected. Two DNA-protein complexes, C1 and C2, were detected. However, only C1 appeared to be specific because formation of the complex was eliminated in the presence of a 50-fold molar excess of unlabeled specific oligonucleotide (Fig. 5A, lane 6). At higher concentrations, a DNA element containing a single copy of the consensus AAAGTTT motif (SIN) was able to abolish almost completely the formation of the C1 complex (Fig. 5A, lane 3) or immune sera against NZF-3 (Fig. 5A, lane 6). The radiolabeled SIN element, however, was unable to form a detectable DNA-protein complex with the whole cell extract (data not shown). This may be explained by the low affinity of NZF-3 for the SIN element and because higher concentrations of NZF-3 are required for the binding to be detected. Indeed, when a bacterially expressed GST fusion protein containing the four-zinc finger cluster of NZF-3 (GST-4ZF) was used in the binding analysis, specific DNA-protein complexes with the SIN element were formed (Fig. 5B). Consistent with the observation from binding analysis with the full-length protein, more avid binding was detected with the DR5 than the SIN element when identical amounts of the bacterial protein were used (Fig. 5B, left and center lanes).

Because an apparent difference was noted in the affinity of NZF-3 binding to the single (SIN) versus the double copy (DR5) site, we decided to evaluate this difference quantitatively by carrying out competitive DNA binding experiments. The relative effectiveness of increasing concentrations of various unlabeled oligonucleotides in competing with a constant concentration of radiolabeled DR5 for binding to NZF-3 was evaluated. The fraction of maximal binding (defined as the ratio of probe shifted in the presence of competitor to that in the absence of competitor) was plotted against the concentration of unlabeled oligonucleotide required to reduce the quantity of the shifted protein-DNA complex by 50%, i.e. IC50, was estimated from the graph and was taken as an indication of the relative binding affinity. The IC50 for the DR5 and SIN was estimated to be 2 nM and 90 nM, respectively. An example of a competition experiment used to determine the relative binding affinity of NZF-3 for the DR5 oligonucleotide is shown (Fig. 5D).

Remarkably, the relative affinities of NZF-3 to direct repeat elements with different spacing (DR1, DR5, DR7, DR9, and DR11) were essentially identical, with less than a 2-fold difference among the elements, regardless of spacing configuration (data not shown).

Transrepression Activity of NZF-3—Both X-MyT1 and NZF-1 were shown to possess transactivation activity from consensus elements in transient transfection analysis (16, 18). To determine the effect of NZF-3 on transcription, transient transfection analysis was carried out in 293 cells with luciferase reporter constructs containing either the single copy (SIN) or the various bipartite elements fused upstream to a herpes simplex
virus thymidine kinase promoter. Reporter constructs containing the various DNA elements exhibited basal promoter activity similar to that containing only the thymidine kinase promoter, suggesting the absence of an endogenous source of activation activity working through these elements in 293 cells. Surprisingly, overexpression of NZF-3 did not result in transactivation of the reporter constructs tested. Expression of the full-length protein was confirmed by Western blot analysis (data not shown). In contrast, overexpression of NZF-3 resulted in a repression of the basal promoter activity (ranging from 40 to 70%) from the reporter constructs containing the bipartite elements with various spacing (data not shown). The most significant inhibition (69%) of the basal promoter activity was observed from the DR9 element. The repression by NZF-3 occurred in a dose-dependent manner (Fig. 6A) and appeared to be binding site-dependent as it did not repress the thymidine kinase promoter alone or the thymidine kinase promoter fused to the p53 or the thyroid hormone response elements (Fig. 6A).

To define the region of the protein required for mediating the repressor activity, a series of deletion mutants was constructed and used in transient transfection analysis. All of the mutants were expressed to a similar extent as determined by Western blot analysis (data not shown). Binding analysis revealed that all of the mutants were competent for DNA binding with the exception of the mutant M6 (data not shown). Deletion of the amino and carboxyl terminus of NZF-3 (mutants M1 and M2) had no significant effect on its repressor activity. Further deletion of either one of the two zinc finger clusters (M3 and M4) also failed to affect the repressor activity. However, the deletion of the inter-zinc finger region (mutant M5) abolished the repressor activity of NZF-3 completely. Mutants M6 and M7, consisting of only the inter-zinc finger region and the distal zinc finger cluster, respectively, also failed to exhibit repressor activity. Inactive mutants that lacked the repressor activity could still be detected in the nucleus (data not shown), suggesting that the loss of repressor function is not caused by changes in subcellular localization. These data indicated that neither the amino nor the carboxyl-terminal domain of NZF-3 is important for the repressor activity. The inter-zinc finger region in combination with either one of the two zinc finger clusters appears to represent the minimum requirement for the activity.
DISCUSSION

We have identified and characterized NZF-3, the third member of the emerging NZF/MyT gene family. The full-length cDNA of NZF-3 encodes a protein of 1,032 amino acids with six zinc fingers of the C2HC type. Similar to other members of this family, NZF-3 is expressed primarily in the nervous system. PCR studies failed to identify any other novel member in non-neuronal tissues, which offers support to the idea that this is a neural specific gene family. The most conserved motif found among members in this family is the zinc finger. The spacing between the cysteine and histidine residues within a finger predicts that the zinc fingers in NZF-3 would be likely to fold into a structure closely resembling that of the C2HC Krüppel type of zinc finger rather than the C2HC fingers of the gag-derived retroviral proteins (9). However, unlike the Krüppel zinc fingers where the most highly conserved feature is the inter-zinc finger linker region, the most conserved amino acids in the finger domain of this family of proteins are those between the first two invariant cysteines and those between the histidine and last cysteine.

DNA binding studies showed that each zinc finger cluster can interact with DNA independently. Members of many zinc finger gene families are found to bind to identical or highly similar nucleotide elements. Examples are members of the NGF1-A family (25), the GATA family of transcription factors (26), and also the family of proteins homologous to erythroid Krüppel-like factor (27). Given that the zinc finger domains represent the most conserved region among members of the NZF/MyT family, it is not surprising that they show striking similarities in their DNA binding profile. Like MyT1 and NZF-1, NZF-3 was found to recognize the consensus AAAGTTT element. However, a bipartite element demonstrated a markedly higher binding affinity to NZF-3. The cooperativity in binding was observed with the full-length protein as well as the isolated zinc finger cluster (GST-4ZF). The mechanism underlying the cooperative binding of NZF-3 to the bipartite element is currently unknown.

That members of this family share similar DNA recognition profiles and expression patterns raises the question of whether they regulate a similar set of target genes. Several possibilities exist. Although these proteins recognize identical sites, differences in their optimal binding affinity for closely related recognition sequences may allow them to regulate genes differently in vivo. In association with differential binding affinity, the DNA binding affinity of these proteins can also be modulated by post-transcriptional modification such as phosphorylation. Examples of proteins that exhibit differential binding activity as a result of their phosphorylation status are the cAMP response element binding protein (CREB) (28) and the serum response factor (SRF) (29). Furthermore, phosphorylation is known to affect the transactivation activity of certain proteins as in the case of Oct-2, which transactivates preferentially from certain promoters on which Oct-1 has no effect even though both proteins apparently recognize the same consensus sequence (30). Lastly, individual members may each interact with different accessory factors that can control their activities in a cellular or developmentally regulated fashion.

Under the conditions tested, NZF-3 appeared to have an inhibitory effect on the basal transcription activity of reporter constructs containing the bipartite DNA-binding elements. It is possible that NZF-3 was displacing a nuclear factor that is capable of binding to and transactivating from the same consensus site as NZF-3. Indeed, NZF-3 competed with NZF-1, another member of the NZF/MyT family of zinc finger proteins, in vitro for binding to the DR9 element (data not shown). Furthermore, overexpression of NZF-3 antagonizes the 2–3-fold activation of the DR9 reporter rendered by overexpression of NZF-1 (data not shown). In principle, NZF-3 can mediate its repression effect by simply displacing NZF-1. However, because both MyT1 and NZF-1 are unlikely to be expressed to any appreciable level in 293 cells, the observed repressor activity is unlikely to be accounted for on the basis of competition with MyT1 and NZF-1 by NZF-3. Moreover, the lack of activation of the basal promoter activity from the bipartite element in the transfection experiments argues against the presence of endogenous activator molecules working through the DR9 element in 293 cells. Hence, a simple displacement model is inadequate to account for the repression activity of NZF-3. In addition, the repressor activity of NZF-3 required the inter-zinc finger cluster domain as well as the DNA binding domain. It is likely that both protein–protein interaction and DNA binding mechanisms are involved in mediating the repressor activity. Because NZF-3 is expressed mainly in neuronal tissues, a fuller understanding of its role in transcription can only be achieved when further studies can be carried out in appropriate neuronal models.

Amino acid sequence analysis of MyT1 and NZF-1 revealed that both proteins contain acidic and serine/threonine-rich domains that are absent in NZF-3. The acidic domains in GAL4 (31) and VP16 (32) and serine/threonine-rich regions in Pit-1/GHF-1 (33) and Egr1 (34) have been demonstrated to serve transactivation functions. Thus, there is a strong likelihood that these domains may contribute to the transactivation activity associated with NZF-1 and MyT1. The absence of these motifs in NZF-3 coupled with the observation that NZF-3 possesses repressor activity in a site-dependent manner permit us to hypothesize that NZF-3 may represent a negative regulator in pathways served by members of this family.

The cloning of the third member of the NZF/MyT gene family thus provides an additional opportunity to examine the relative contributions of members of this family of neural specific transcription factors in the development and homeostasis of the nervous system.

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