A Novel Family of Cys-Cys, His-Cys Zinc Finger Transcription Factors Expressed in Developing Nervous System and Pituitary Gland*

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A screen designed to identify proteins that specifically bind to retinoic acid response elements resulted in the identification of a rat cDNA encoding a novel protein containing six Cys-Cys, His-Cys zinc fingers. This gene is expressed in a restricted fashion exhibiting distinct temporal and spatial patterns in the developing nervous system, primarily brain, spinal cord, sensory ganglia, retina, and nasal epithelia, as well as in the pituitary, and is referred to as neural zinc finger factor 1 (NZF-1). NZF-1 binds specifically to a cis-regulatory element of the β-retinoic acid receptor (RARβ) gene, as well as to other related DNA elements, including two in the upstream enhancer region of the mouse Pit-1 gene. In heterologous cells, NZF-1 activates transcription from promoters containing specific binding sequences and can synergize with other factors, such as Pit-1, to regulate gene expression. These results suggest that NZF-1 may exert regulatory roles in the developing and mature nervous system and in the pituitary gland. Identification of a second mouse gene highly homologous to NZF-1, encoded by a distinct genomic locus, reveals a dispersed gene family encoding proteins containing Cys-Cys, His-Cys motifs.

Precise temporal and spatial patterns of development are controlled by sequential activation of a hierarchy of regulatory genes, which encode transcription factors containing multiple classes of DNA binding motifs. Zinc coordinated fingers are one of the most common DNA binding motifs among eukaryotic classes of DNA binding motifs. Zinc-coordinated fingers are one of the most common DNA binding motifs among eukaryotic classes of DNA binding motifs. These proteins contain two or more fingers in a tandem repeat. In contrast, steroid receptors, such as the glucocorticoid receptor, contain only two zinc coordinated structures with four (C4) and five (C5) conserved cysteines. The third class of zinc fingers, which also binds to single-stranded nucleic acids, has a consensus sequence of Cys-X2-Cys-X2-His-X2-Cys. Such factors are found in transposable element copia, plants, and mammalian cells as well as in retroviruses. Other metal-coordinating proteins have different structures such as Cc in the yeast GAL4 protein and a cysteine-rich structure in the E1A oncoprotein (2).

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 SPI are broadly involved in the regulation of transcription, whereas the Drosophila zinc finger proteins Kruppel and Hunchback are crucial for proper segmentation of the developing embryo (3–5). In humans, mutations in a kidney zinc finger protein (WT1) result in Wilms’ tumor (6, 7). Recently, a zinc finger protein (REST) has been shown to repress neuronal gene expression in non-neuronal tissues (8, 9).

Because retinoic acid receptor (RAR) binds ineffectively to DNA response elements, requesting a DNA binding co-regulator, expression screening was performed with a β-retinoic acid response element (βRARE). This proved to be a successful strategy for the cloning of a retinoic acid receptor co-regulator which was identified as a member of the retinoid X receptor family (10–12). This co-regulator binds DNA cooperatively as a heterodimer with the retinoic acid receptor and other nuclear receptors (11–16).

In order to investigate the mechanisms that underlie regulation of the expression of the β-retinoic acid receptor (RARβ) and Pit-1 genes further, we screened expression libraries based on the detection of protein-DNA interactions using the βRARE and the retinoic acid response element of the Pit-1 gene enhancer (17). In this manuscript we report that, as a consequence of these screens, we have identified a novel transcript encoding a Cys-Cys, His-Cys zinc finger protein. Based on its restricted expression pattern in the nervous system, this factor is referred to as NZF-1 (neural zinc finger factor 1). Characterization of NZF-1 has revealed the presence of two separate Cys-Cys, His-Cys type zinc finger DNA binding domains, each

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The abbreviations used are: RAR, retinoic acid receptor; βRARE, β-retinoic acid response element; NZF-1, neural zinc finger factor 1; bp, base pair(s); kb, kilobase(s); RFLV, restriction fragment length variant; GST, glutathione S-transferase; RSV, Rous sarcoma virus; CMV, cyto-megalovirus; PSE, pituitary specific element.

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of which can bind independently to similar DNA sequences. The preferred consensus sequence is distinct from the consensus retinoic acid receptor response element. The NZF-1 gene exhibits a restricted pattern of expression in the nervous system, testis, and pituitary gland. NZF-1 can serve as a transcription factor and elicit synergistic activation with other factors, such as Pit-1. Our results suggest that this novel zinc finger protein may exert roles both in the development and function of the central nervous system and the anterior pituitary gland.

MATERIALS AND METHODS

Cloning of NZF-1—A concatenated bRARE oligonucleotide (5'-AATTGGGTTCCAGCAAGTTGTCAC-3') was used to screen a rat pituitary G cell λgt11 expression library as described previously (18). Multiple, overlapping NZF-1 clones were subsequently isolated from rat cerebellum and pituitary cell line cDNA libraries by DNA hybridization screening. cDNA inserts were subcloned into pBR322(-) (Stratagene) and sequenced as double-stranded templates using a Sequenase 2.0 kit (U. S. Biochemical Corp.).

RNase Protection Assays and in Situ Hybridization Analysis—RNase protection assays were performed using 32P-radiolabeled probes, as described previously (19). Hybridization reactions contained 10 μg of yeast tRNA or total tissue RNA. A rat-actin probe (280 nucleotides) was included as a control when indicated. Ribonuclease probes were transcribed in vitro using constructs containing a 610-bp fragment of the NZF-1 cDNA coding for amino acids 446–484 (probe A) or a 390-nucleotide fragment coding for amino acids 753–882 (probe B) as templates.

For in situ hybridizations, cryosections of rat embryonic tissues were mounted on poly-L-lysine-coated slides and air-dried. Pretreatment, hybridizations, and washing conditions have been described (20). Briefly, sections were digested with proteinase K (10 μg/ml, 37 °C, 30 min), acetylated, dehydrated, and dried. 50 μl of hybridization mixture containing 32P-labeled probe (106 cpm/ml) was spotted on each slide. After 15 h at 55 °C for 16 h, slides were rinsed, digested with ribonuclease A (20 μg/ml, 37 °C, 30 min), and washed in 0.1× SSC for 30 min at 65 °C. After dehydration, slides were dipped in Kodak NTB2 autoradiographic emulsion (diluted 1:1 with distilled water), dried in a humid chamber (2–4 h), exposed desiccated in the dark at 4°C for two weeks, and developed with Kodak D-19, followed by Autoradiographic Fixer.

Genomic Southern Blot Analysis and Mouse Chromosomal Localization—Duplicate samples (10 μg) of mouse and rat genomic DNA were digested separately with restriction endonucleases BamHI, BglII, and SacI, electrophoresed through a 0.7% agarose gel, and transferred to a nylon membrane. The radiolabeled probes used were a 400-bp fragment from rat NZF-1 and a 1.2-kb fragment from mouse NZF-2. Hybridizations were carried out in 2× sodium dodecyl sulfate, 0.1× SSC, 5× Denhardt's solution, and 100 μg/ml carrier salmon sperm DNA at 65 °C. After washing under high stringency conditions (0.3× SSC at 65 °C), the membrane was autoradiographed.

The mapping of the genes for Nsf-1 and Nsf-2 in mouse was performed by linkage analysis of an interspecific backcross. Progeny from a (C57BL/6J × Mus spretus) F1 × C57BL/6J backcross were typed for 10 different restriction enzymes followed by Southern analysis. A 1060-bp XhoI-BamHI fragment from the rat NZF-1 cDNA and a 1.3-kb XhoI fragment from the mouse NZF-2 cDNA were utilized as probes. Blots were hybridized with 32P-labeled probes at 65 °C and washed in 1.0× SSC, 0.1% SDS at 50 °C essentially as described previously (21).

Protein Overexpression, Electrophoretic Mobility Shift Assays, and DNA Methylation Interference Analysis—In order to study the in vitro DNA binding properties of this factor, fragments of the NZF-1 cDNA were subcloned into the pGEX-KG vector (22) and GST fusion proteins were expressed in Escherichia coli BL21 (DE3). Construct 2ZF was generated by subcloning a 990-bp NcoI-XhoI fragment of cDNA clone 13A into the corresponding sites of pGEX-KG, while 1ZF was constructed with an equivalent NcoI-XhoI fragment from clone 3B which has a deletion of 135 nucleotides encoding a zinc finger (Fig. 1B). An additional 1.4-kb XhoI-XhoI fragment encoding the C-terminal portion of NZF-1 was introduced into 2ZF to make 5ZF. 3ZF was generated by subcloning a polymerase chain reaction fragment encoding three C-terminal zinc fingers into the pGEX-KG vector. In electrophoretic mobility shift assays, purified GST fusion proteins were preincubated in 20 μl of binding buffer (50 mm HEPES, pH 7.9, 50 mm KCl, 5% glycerol, 2 mg/ml bovine serum albumin, 10 μg/ml ZnSO4) containing 1 μg of poly(dI-dC) and 0.1 μg of salmon sperm DNA for 5 min. Samples were incubated for an additional 25 min at room temperature after adding 32P-radiolabeled probe (0.1–0.5 ng). 4.5 μl of each reaction was loaded onto a 5% nondenaturing 0.5 × TBE polyacrylamide gel and electrophoresed at 20 V/cm for 60 min. Gels were dried and autoradiographed.

For methylation interference experiments, oligonucleotides were labeled at the 5′ end using T4 kinase and γ32P-ATP. After annealing, double-stranded probes were gel-purified and methylated with dimethyl sulfate. Binding reactions were carried out as described above. Free and protein-bound DNAs were separated by electrophoresis on nondenaturing polyacrylamide gels and subjected to pipieridine cleavage after elution. Cleaved products were separated on 12% polyacrylamide gels containing 7 m urea.

Cloning of a Neural-specific Zinc Finger Protein—The 500-bp mouse Pit-1 enhancer/promoter/luciferase reporter plasmid has been described (17). A 300-bp HRAR promoter/luciferase reporter, CMV-Pit-1, and RSV-hRARα have been described previously (23, 24). African green monkey kidney cells (CV-1) were plated at a density of 2 × 105 cells per 60-mm plate in Dulbecco's modified Eagle's medium containing 10% newborn calf serum. 24 h later, cells were transfected with 2 μg of reporter plasmid and 1 μg of expression plasmid using the calcium phosphate co-precipitation method (25). Cell extracts were prepared and luciferase assays were performed as described (26).

RESULTS

Cloning of a Full-length cDNA and Splice Variants of NZF-1—Using radiolabeled bRARE DNA binding site as a probe, a 1.3-kb cDNA fragment was cloned from a rat pituitary cell line (GC) λgt11 library by expression screening. Sequence analysis of this clone revealed the presence of an open reading frame containing two Cys-Cys, His-Cys type zinc fingers, which would be predicted to form a DNA binding motif. Because the 5′ end of this cDNA did not contain an initiator methionine, five additional overlapping clones, two from rat pituitary cell line libraries, and three from a rat cerebellum library were obtained by DNA hybridization screening and sequenced (Fig. 1B), all proving to represent sequences from a single transcript. The assembled 4.7-kb NZF-1 cDNA sequence contained stop codons in all three reading frames preceding an ATG that initiated a 3.5-kb open reading frame, followed by a 360-bp 3′-untranslated region containing consensus polyadenylation signals.

The sequence predicted an 1187-amino acid protein that contained six Cys-Cys, His-Cys type zinc fingers, organized in clusters of two and three zinc fingers, separated by 329 amino acids as well as a single zinc finger near the amino terminus (Fig. 1A). The amino acid sequence of the six zinc fingers share striking homology with each other (Fig. 1C). The NZF-1 protein contains a highly acidic region in the amino terminus (79% Asp or Glu, between amino acids 88 and 173), and a serine/threonine-rich sequence located between the two clusters of zinc fingers (35% Ser or Thr, between amino acids 625 and 715) (Fig. 1A).

We subsequently identified two alternatively spliced forms of NZF-1, which predict deletions of 63 and 135 nucleotides, removing coding information for the first and second zinc fingers, respectively. In an RNase protection assay, one of the splice forms is expressed at levels significant when compared to the predominant transcript encoding the larger 1187-amino acid protein (Figs. 1B and 2A and data not shown).

Neuronal-restricted Expression of NZF-1—Ribonuclease protection assays were employed to study the tissue distribution of NZF-1 messenger RNA. A wide variety of rat tissues were examined, including muscle, kidney, spleen, liver, heart, brain, skin, lung, eye, thymus, pancreas, adrenal, testis, and ovary. NZF-1 mRNA was detected only in brain, adult pituitary, and pituitary cell lines (Fig. 2A). Because NZF-1 expression in testis was detected with a probe encompassing nucleotides...
2979–3366 (probe B), but not nucleotides 2058–2664 (probe A), an alternatively spliced form of NZF-1 appears to be selectively expressed in the testis (Fig. 2B). This conclusion is consistent with RNA blot analysis, which revealed that transcripts detected in brain and testis using NZF-1 probe are of different size (data not shown). The levels of NZF-1 gene expression became progressively higher in adult as compared to neonatal pituitary (Fig. 2B), and the levels of NZF-1 expression were consistently higher in brain than those in pituitary and testis.

In order to determine the temporal and spatial expression patterns of NZF-1, we performed in situ hybridization experiments on both embryonic and adult rat tissues. As early as e11.5, NZF-1 expression was evident throughout the proliferating cortex neuroepithelium, the developing medulla, and in
the spinal cord. At e12.5, NZF-1 mRNA was detected in the nasal epithelium as the nasal cavity forms (data not shown). At e13.5, NZF-1 expression was detected in the trigeminal ganglia, the dorsal root ganglia, and the ganglion cell layer of the retina (Fig. 3A). The expression of NZF-1 transcripts in the brain and the spinal cord reached the highest levels of detection at e14–e15 and subsequently decreased slightly (Fig. 3 and data not shown). Later in brain development, transcripts of NZF-1 were detected in the cerebellar neuroepithelium, choroid plexus primordium, and within the cephalic flexure. NZF-1 transcripts were widely expressed in the adult brain, at levels lower than that detected during development. In situ hybridization experiments revealed that in adult testis, NZF-1 was expressed in the periphery of a subset of seminiferous tubules, indicating that the mRNA was present at early stages of the developing germ cell, i.e. in spermatogonia and/or early phase of spermatocytes I (data not shown). Thus, expression of the NZF-1 gene is restricted to the nervous system, pituitary gland, and testis, exhibiting highest levels of expression in the developing central nervous system.

NZF-1 Is a Member of a Gene Family—Interestingly, an NZF-1 cDNA was also isolated by expression screening with a Pit-1 enhancer element (RDE) (17) from a pituitary cell line (GC) λgt11 library, performed in an effort to search for transcription factors that interact with the Pit-1 gene enhancer. A second element in the Pit-1 gene enhancer, the pituitary specific element (PSE) (17), was used as a probe in expression screening of a mouse pituitary library, resulting in the identification of a cDNA encoding a highly related mouse zinc finger protein, referred to as NZF-2. Sequencing of fragments encompassing this clone revealed that it contains five zinc fingers corresponding to specific fingers in NZF-1 (Fig. 4A). This mouse clone has high homology to the human glia transcription factor referred to as MyT1, reported to bind to the proteolipid protein gene promoter (27).

Genomic Southern blot analysis was employed to determine whether NZF-1 and NZF-2 were encoded by distinct genes. Duplicate Southern blots hybridized with cDNA probes encoding homologous regions of NZF-1 and NZF-2 revealed distinct patterns for the two probes, indicating they are encoded by two
different genes (Fig. 4B). The chromosomal localization of the two NZF genes were determined in mouse by linkage analysis of a backcross between strains C57BL/6J and M. spretus, with C57BL/6J as the recurrent parent. A screen of restriction enzymes revealed a restriction fragment length variant (RFLV) for the NZF-1 gene, designated Nzf-1, with the enzyme PvuII. Following Southern analysis, DNA from strain C57BL/6J yielded hybridized bands of 8.0, 5.2, and 2.7 kb, DNA from M. spretus yielded bands of 9.8 and 8.0 kb, and DNA from F1 hybrids contained all these bands (data not shown). The RFLV was scored in 49 backcross mice and compared to the segregation of about 350 previously typed genetic markers. Linkage analysis indicated that the AAGTT sequence was crucial for optimal binding and that purines at the two bases preceding this element revealed the critical nucleotides of GAAAGTT (Fig. 6B). Therefore we conclude that for all elements examined, the core DNA element recognized by NZF-1 was GAAAGTT.

In order to determine the specific nucleotide sequences that were critical for binding by these two or three zinc finger regions, we utilized synthetic double-stranded oligonucleotides encompassing wild-type and mutant bRARE using electrophoretic mobility shift assays. Systematic mutations altering this element revealed the critical nucleotides of GAAAGTT motif for binding by the 2ZF polypeptide. The region encompassing these three zinc fingers bound an additional GTT motif 4 bp 5' of the GAAAGTT core (Fig. 6A). Although the peptide containing one zinc finger would still be predicted that this protein would contain two clusters of zinc fingers capable of serving independently as DNA binding domains. We examined bacterially expressed glutathione S-transferase fusion proteins containing either the central region with one or two zinc fingers (1ZF and 2ZF), a carboxy-terminal fragment spanning three zinc fingers (3ZF), or a region of NZF-1 spanning five zinc fingers (5ZF) (Fig. 6A).

FIG. 5. Distinct chromosomal loci for Nzf-1 and Nzf-2. Using an interspecific backcross ((C57BL/6J × M. spretus) F1 × C57BL/6J), Nzf-1 and Nzf-2 were mapped to chromosomes 12 and 2, respectively. The ratios of the number of recombinations to the total number of mice and the recombination frequencies ± S.E. (in centimorgans) for each pair of loci, are indicated in the left of the chromosomes. For pairs of loci that co-segregate, the upper 95% confidence interval is shown in parentheses. All loci were linked with lod scores greater than 8.0. Chromosomes are drawn to scale with the distance of the most distal marker from the centromere (top) given below each chromosome in centimorgans. Markers were reported in Ref. 21 or are unpublished data.
sequence are preferred for optimal binding by the NZF-1 zinc fingers (Fig. 7).

Electrophoretic mobility shift experiments confirmed that NZF-1 bound specifically to a series of DNA sites: Pit-1 PSE, Pit-1 RDE, and the pyridoxal phosphate promoter element (data not shown) in addition to the βRARE, and all four DNA elements used in expression cloning shared a highly homologous core motif (Fig. 7). NZF-1 and NZF-2 share a high degree of amino acid similarity in the zinc finger regions as well as in the carboxyl terminus and bind to the same sequences.

Analysis of methylation interference of binding by the methyl groups of the N-7 position of guanine and the N-3 position of adenines, indicative of major and minor groove contacts, respectively, was then utilized to investigate the specific contacts of the NZF-1 protein with target DNA elements. 32P-Labeled probes (representing the βRARE and the Pit-1 PSE) were methylated with dimethyl sulfate and incubated with GSTNZF-1. The pattern of inhibition of G residues in the major groove of the consensus core motifs and of A residues showing weaker signals in the bound complexes indicated that the core binding sequence was consistent with that defined by mutagenesis, electrophoretic mobility shift assays (Fig. 8).

**Transactivation by NZF-1**—In addition to DNA binding motifs, NZF-1 contained regions such as acidic and serine/threonine-rich sequences that have been commonly found in the activation domains of many transcription factors. CV-1 and HeLa cells, which do not contain detectable NZF-1 mRNA by RNase protection assay, were chosen to examine the potential transcriptional effects of NZF-1. Because NZF-1 was found to bind to both βRARE and Pit-1 enhancer elements in a sequence-specific manner, we tested whether NZF-1 was capable of transactivating promoters containing these elements. The complete NZF-1 cDNA was introduced into an expression vector under control of the Rous sarcoma virus promoter and co-transfected with the hRARβ promoter-luciferase reporter plasmid. NZF-1 moderately activated the hRARβ promoter, approximately 3-fold compared with the RSV vector control (Fig. 9A). When NZF-1 was co-transfected with a Pit-1 enhancer/promoter luciferase reporter gene in HeLa cells, NZF-1 activated the reporter 4-fold, and co-transfected NZF-1 synergized with Pit-1 (Fig. 9B). Thus, in this context, the NZF-1 protein is capable of serving as a co-regulator. Retinoic acid can regulate gene expression through the action of its receptors. However, NZF-1 neither synergized with retinoic acid receptor nor inhibited activation by retinoic acid of the hRARβ gene promoter (data not shown). It therefore appears that NZF-1 and retinoic acid can independently activate the hRARβ promoter.
DISCUSSION

We have identified a 4730-base pair cDNA encoding a novel 1187-amino acid protein, NZF-1, containing six Cys-Cys, His-Cys zinc fingers, organized as internal clusters of two and three fingers, with one finger located in the amino terminus. The expression of this factor is highly restricted to the nervous system, pituitary gland, and testis with highest levels of expression in the developing nervous system.

The primary amino acid sequences of all component zinc fingers are very homologous. Because of the spacing between the Cys and His residues, the folding of the NZF-1 zinc finger motifs is likely to more closely resemble that of the Cys-Cys, His-His type of zinc fingers than the Cys-Cys, His-Cys fingers of retrovirus proteins, such as the nucleic acid-binding protein of Rauscher murine leukemia virus (28).

Each zinc finger cluster forms an independent DNA binding domain and recognizes similar specific target sequences, although the three zinc finger domain demonstrates higher binding specificity. Crystallographic studies on the Cys-Cys, His-His type of zinc fingers than the Cys-Cys, His-Cys fingers of retrovirus proteins, such as the nucleic acid-binding protein of Rauscher murine leukemia virus (28).

The genes for NZF-1 and NZF-2, designated Nzf-1 and Nzf-2, respectively, were mapped in the mouse by linkage analysis of an interspecific cross. Nzf-1 is located on mouse chromosome 12 in a region that appears to be syntenic with human chromosome 2p. Nzf-2 is located on distal mouse chromosome 2 in a region syntenic with human chromosome 20q. Neither region in the mouse contains mapped mutations that are likely to be relevant to these genes.

Thus, a new family of Cys-Cys, His-Cys proteins, expressed
in the nervous and endocrine systems, has been identified, and these factors are likely to exert selective functions in development and homeostasis of the neuroendocrine system.

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