

Differential splicing creates a diversity of transcripts from a neurospecific developmentally regulated gene encoding a protein with new zinc-finger motifs

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ABSTRACT

We have cloned a novel neurospecific gene, named neuro-d4, by differential screening a rat cerebral cortex cDNA library. Northern blot hybridization showed that neuro-d4 expression is restricted to neuronal tissues both in newborn and adult animals. The level of neuro-d4 mRNA in the rat central nervous system is high during the later stages of embryonic development and gradually decreases during the postnatal period. *In situ* hybridization suggests that the gene transcripts are localized in neuronal cell bodies. Nucleotide sequences of overlapped cDNA clones and all 12 exons in genomic clone were determined. The deduced protein has consensus sequences for a nuclear localization signal, a Krüppel-type zinc-finger and a new type of cysteine/histidine-rich motif resembling zinc-fingers. Several differential splicing variants were found, each of which influences the structure of the encoded protein.

INTRODUCTION

Extensive functional changes occur in the central nervous system during the early postnatal period. To identify novel genes active during this stage of central nervous system development we differentially screened a 7–9 day rat cerebral cortex cDNA library and isolated clones corresponding to neurospecific genes with higher levels of expression in the nervous system of young rats. The present paper is concerned with one of these genes encoding a novel zinc-finger protein.

These proteins bind nucleic acids and have common sequence units, termed zinc-fingers, which contain clusters of cysteines or cysteines and histidines that coordinately bind zinc ions. Some amino acid consensus sequences of these domains have been characterized and correlations between structure, nucleic acid binding specificity and function have been proposed (1, 2). Zinc-finger proteins are widespread and participate in the assembly of virus particles (3, 4), embryogenesis (5–7), sex determination

(8) and cell proliferation (9, 10). Some zinc-finger proteins play a role in the development of the nervous system (11–13). However, most of the known genes encoding these proteins are expressed in a variety of tissues, indicating a broad role in the general events of differentiation rather than in events specific to neurogenesis.

Here we report the molecular cloning, structure, diversity and pattern of expression of a gene, named neuro-d4, that encodes a new type of zinc-finger protein. In contrast to most previously identified zinc-finger genes, the expression of neuro-d4 is not only restricted to the nervous system but also appears to be found only in certain neurons.

MATERIALS AND METHODS

Cloning procedures

Polyadenylated RNA from 7–9 day rat cerebral cortex was used to construct a cDNA library in lambda gt10 vector according to Amersham kit protocol. Three replicas of the 50,000 clones from the library were screened with high specific activity cDNA probes. These probes were prepared by reverse transcription of polyadenylated RNA from P7–9 cortex, adult cortex and adult liver. A clone named d4 gave strong, weak and no signal with these probes, respectively. Three rounds of plating and hybridization were done to confirm these differences and to purify the clone. Clone lambda d41G was isolated from a rat EMBL4 genomic library, constructed as described by Maniatis et al. (14), by hybridization with a nick-translated 0.85 kb. insert of the d4 clone as a probe. Twelve cDNA clones designated with letter 'C' were isolated from the same cDNA library by hybridization with a nick-translated fragment of the genomic clone corresponding to exons XI and XII or to exon I (for clone 8C). Clone RACE H12 was obtained as described by Frohman et al. (15) using specific oligonucleotide 5'-TCCCGGTAGAAGTCC-TCGCCAAGGCTCAGGGGGCTG-3' and dG-tailing of cDNA. All mapping and subcloning procedures are described elsewhere (14).

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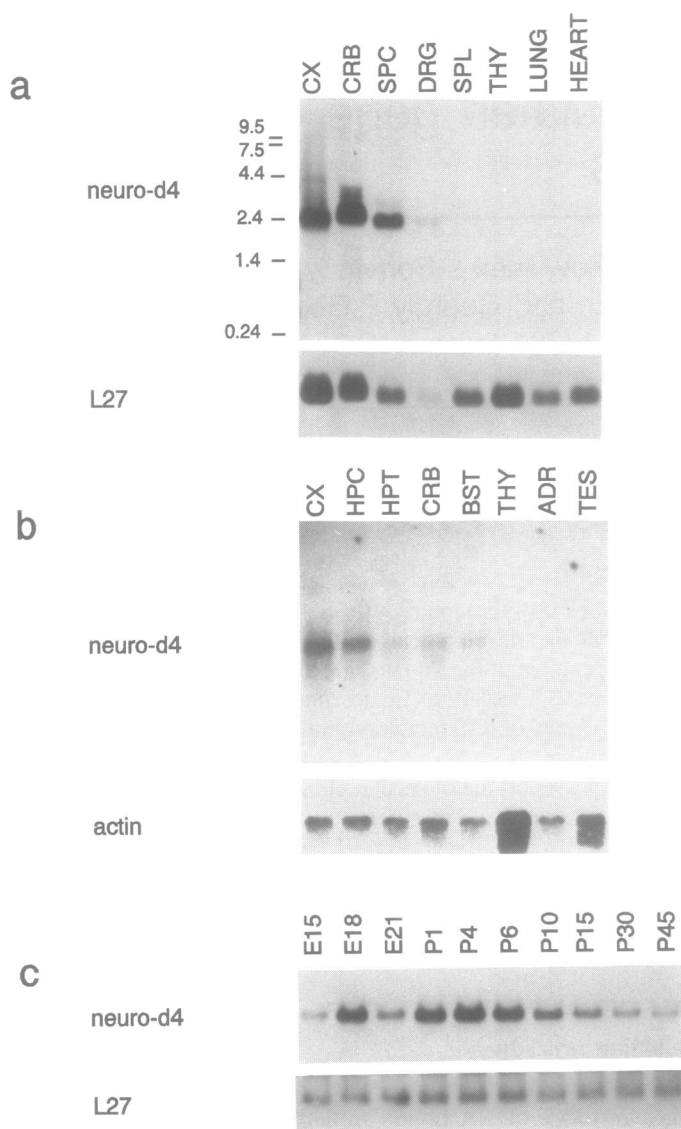


Figure 1. Northern-blot analysis of neuro-d4 expression. a, hybridization of total RNA from different tissues of newborn rats using a nick-translated probe derived from clone 6C. The positions of RNA-ladder markers (BRL) are shown. CX—cerebral cortex; CRB—cerebellum; SPC—spinal cord; DRG—dorsal root ganglion; SPL—spleen; THY—thymus. b, hybridization of total RNA from adult rat tissues using a nick-translated probe derived from clone d4. HPC—hippocampus; HPT—hypothalamus; BST—brainstem; ADR—adrenal gland; TES—testis. c—hybridization of total RNA from whole brains of embryonic (E) and postnatal (P) rats with the same probe as in 'a'.

DNA sequencing

DNA fragments of cDNA clones were subcloned in pGEM-3Z, pGEM-4Z or pBluescript SCII⁺ plasmids and the both strands were sequenced by the chain-termination method using denatured double-stranded templates (16). All exons and parts of introns of the genomic clone were sequenced.

RNA extraction and Northern hybridization

RNAs were extracted from frozen tissues by the guanidinium thiocyanate method (17), and after electrophoresis in 1.2% agarose/formaldehyde gels were blotted to Hybond-N membranes (Amersham). DNA fragments were eluted from LMP-agarose (Gibco-BRL) gels and were labelled by nick-translation.

Hybridization at 42°C in 50% formamide; 5×SSC; 5×Denhardt's solution; 0.5% SDS; 2 mM EDTA; 0.25 mg/ml denatured salmon testis DNA was followed by washing in 2×SSC; 0.2% SDS at 68°C. After removing the hybridization probes, the filters were rehybridised with a mouse beta-actin probe or with a fragment of the rat ribosomal protein L27 gene (18).

In situ hybridization

12 micron cryostat sections were attached to glass slides covered with poly(L)-lysine. Digoxigenin-labelled cRNAs were prepared using the Boehringer Mannheim kit. Hybridization at 57°C in 50% formamide; 4×SSC; 2×Denhardt's solution; 0.5 mg/ml salmon testis DNA; 0.25 mg/ml tRNA *E.coli* was followed by washing in 0.1×SSC at 57°C and detection of hybridized probe with the Boehringer Mannheim detection kit.

RESULTS

Isolation of cDNA and genomic clones

A 7–9 postnatal day (P7–9) rat cerebral cortex cDNA library was differentially screened with three radiolabelled cDNA probes derived from P7–9 cortex, adult cortex and adult liver. A clone, designated d4, was chosen for further study because it hybridized more strongly with 7–9 day cortex probes than with adult cortex probes (see Materials and Methods). An insert of this clone possessed a 44 bp poly(A) tail and had an overall length of 0.85 kb. The presence of the poly(A) sequence together with multiple stop codons in all reading frames indicated that the d4 clone represents part of the 3' untranslated region (3' UTR) of a larger mRNA (named neuro-d4). On Northern blots, a 2.4 kb polyadenylated RNA was detected in nervous tissues using a nick-translated d4 hybridization probe (Fig.1). However, we failed to isolate larger cDNA clones from the library with help of this probe possibly because of strong secondary structure formed by the 3' UTR of the neuro-d4 mRNA. To overcome this problem, we first isolated a 13 kb genomic clone from a rat genomic library, and after mapping and localization of the d4 sequence in this genomic clone, a different hybridization probe was chosen. Screening of 500,000 clones from the same cDNA library with this new probe (corresponding to exons XI and XII, see below) allowed us to isolate 11 further clones that overlapped each other and the original d4 clone (Fig.2). All these clones were primed on the oligo(A) stretch inside the 3' UTR of the neuro-d4 mRNA (see Fig.2 and 3). Clone 8C was isolated by screening the library with the XhoI-BamHI fragment of the genomic clone which corresponds to exon I. To isolate most 5' of the terminal sequence of the mRNA we used the RACE technique with a specific oligonucleotide primer that overlaps the boundary of exons I and II (see Materials and Methods).

Differential splicing of neuro-d4 transcripts

Sequencing of overlapping cDNA and genomic clones revealed a complex pattern of differential splicing of neuro-d4 gene transcripts (Fig.2 and 3). Four splice variants were found, each of which influenced the structure of the encoded protein (see Discussion), but only one of which changed the length of the mRNA significantly. The latter transcript (represented in clones 8C and 18C) has an additional 783 nucleotides from exon I (Fig.2 and 3) and is observed as a weak band above the main one on Northern blots (Fig.1). The identity of this larger transcript was confirmed by hybridization with an exon I-specific probe (data not shown). The differences in length of other neuro-d4

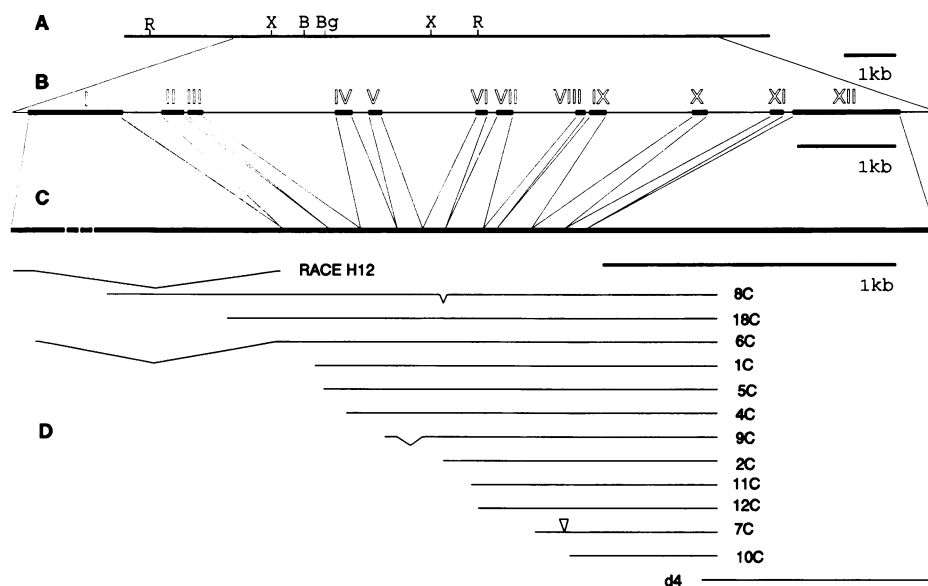


Figure 2. Structure of the neuro-d4 gene and corresponding genomic and cDNA clones. A, physical map of lambda d41G clone. R—EcoRI; X—XhoI; B—BamHI; Bg—BglII. B, intron-exon structure of the neuro-d4 gene. Exons are shown by thick lines and numbered above. C, structure of the longest neuro-d4 mRNA. D, organization of cDNA clones: the deletion of exon V in clone 9C, deletion of part of exon I in clones 6C and RACE H12, deletion of 14bp of exon VI in clone 8C and the insertion of 30 bp of exon XI in clone 7C are shown (see text and Fig.2a).

transcripts are not large enough to separate these transcripts on agarose gels, used for preparing Northern blots. The cDNA clone 9C lacks 90 nucleotides, corresponding to exon V, that are present in the other clones. Clone 7C has a 30 bp insert that results from the use of an alternative upstream acceptor site in intron X. In clone 8C, 14 nucleotides are absent because an upstream donor site was used in exon VI (Fig.2 and 3).

Beside splicing variants, nucleotide substitution was also found in the coding region. In clone 6C, T at position 2023 is substituted by C, resulting transition of Tyr³⁶³ to His³⁶³ (Fig.3). In the 3' UTR at position 2195, T is present in all cDNA clones but it is substituted by C in the genomic clone, confirming the existence of several allelic variants of the neuro-d4 gene in rats.

Neuro-d4 gene encodes a zinc-finger protein

In the most abundant transcript, the first ATG codon (53–55 nt., Fig.3) is located in a appropriate consensus of eukaryotic translation start site (19) and the deduced protein sequence consists of 387 amino acids. Searching nucleic acid and protein sequences data banks revealed only one region of homology to known sequences. Amino acids 193–221 matched perfectly with the consensus sequence of the *Krüppel*-type zinc-finger (Fig.3 and 4a). The usual length of H-H loop in this kind of zinc-finger is three or four amino acids. In the case of neuro-d4, two histidines were found at positions 216 and 218. It was difficult to chose from the sequence data which of these histidines is the Zn²⁺-coordinated amino acid. In the case of His²¹⁸, the length of the H-H loop is four amino acids, but the choice of His²¹⁶ led to better homology between the following amino acids in neuro-d4 and the conserved H-C link of C₂H₂ multifinger proteins (Fig.4a). The finger structure is followed by a stretch of five glutamic acids interrupted by one glycine (Fig.3). Such acidic regions are believed to be an important part of the activating sequences in some transcription factors (22). A basic amino acid domain was found within the N-terminal part of the protein. The

structure of this domain contained a consensus sequence for the nuclear protein localization signal (23).

The C-terminal domain of the neuro-d4 protein contained novel cysteine/histidine-rich sequences resembling fingers. Possible variants of structure of this region are discussed below (see Discussion).

Pattern of neuro-d4 transcription

Different fragments of the neuro-d4 cDNA and genomic clones were used as the probes for Southern, Northern and *in situ* hybridization. All bands revealed on Southern blots correlated with the structure of genomic clones, even in case of moderate stringency hybridization conditions (data not shown), which suggests that the unique neuro-d4 gene does not belong to the family of closely related genes. Northern blot hybridization using these probes showed that neuro-d4 expression is restricted to neuronal tissues both in newborn and adult rats (Fig.1a and b). Neuro-d4 mRNA was detected in the peripheral nervous system (dorsal root ganglia, Fig.1a) as well as in different regions of the central nervous system, where its concentration was highest in hippocampus and cerebral cortex (Fig.1b). The level of neuro-d4 mRNA is abundant in embryonic rat brain and decreases gradually in postnatal development (Fig.1c).

The results of *in situ* hybridization agreed with those of Northern blot hybridization and additionally showed that neuro-d4 expression is restricted to neurons. No or very weak hybridization signals were detected in regions devoid of neuron cell bodies (e.g. in the corpus callosum and the fibrillar layer of the cerebellum, Fig.5). Neuro-d4-positive neurons were observed in the cerebral cortex, hippocampus, several brainstem nuclei and cerebellum (Fig.5a,b,c,e). In the developing cerebellum, strong hybridization was seen in both granule layers (Fig.5e), while after maturation only a weak signal remained in the granule cells and Purkinje cells were intensely labelled (Fig.5c).

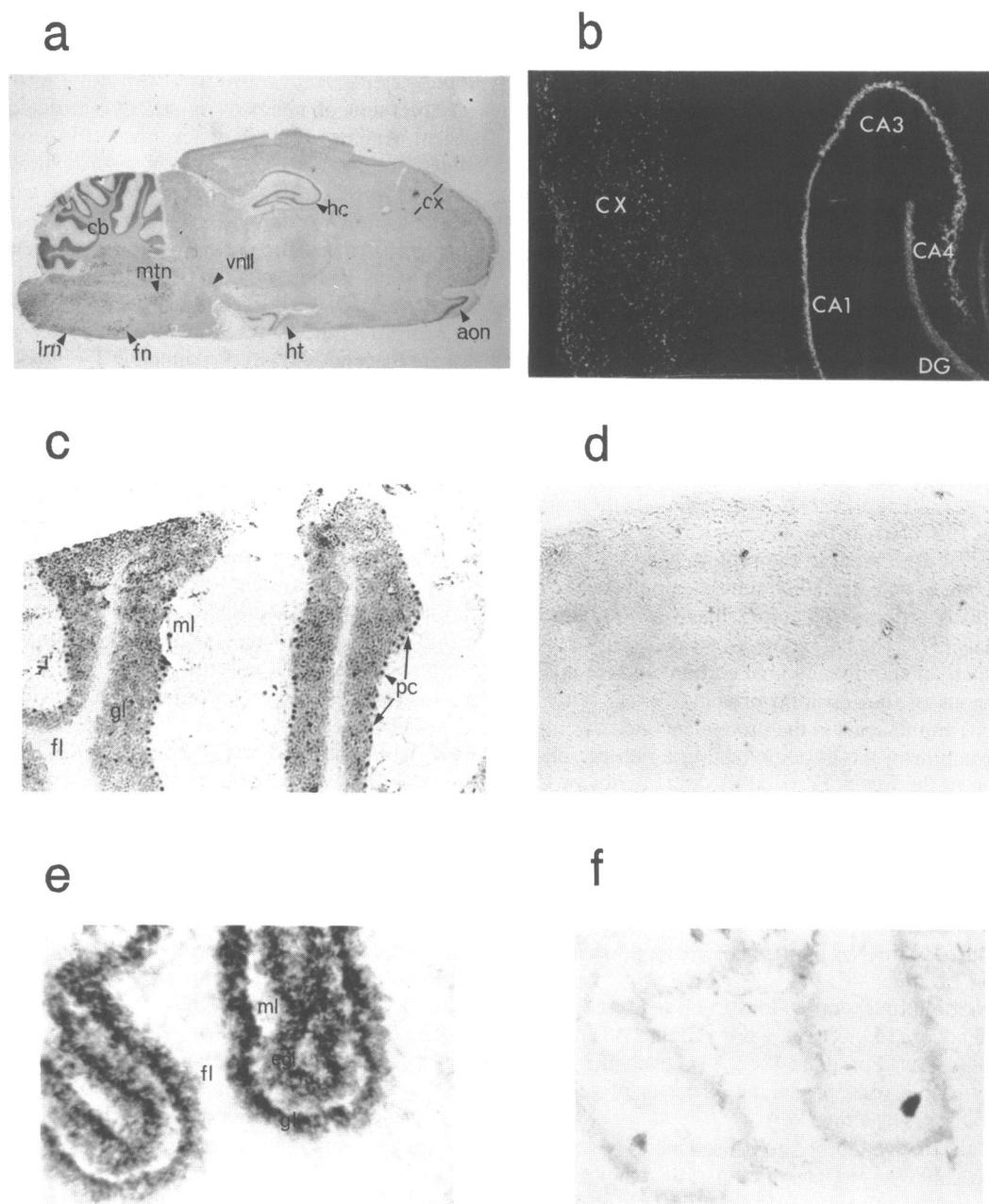


Figure 5. *In situ* hybridization of rat brain sections with digoxigenin-labelled neuro-d4 cRNA probes. a–d, hybridization of adult brain sections with alkali-fragmentated antisense cRNA probe from clone 6C overlapped the entire coding region of the gene. Parasagittal section of adult brain (a); dark field image of the hippocampus region and overlying cerebral cortex (b); part of the cerebellar cortex (c,d). Section shown in d was digested with RNaseA before hybridization. e–f, hybridization of newborn brain sections with antisense (e) or sense (f) cRNA probes from clone 9C (130nt probe overlapping the junction of exons IV–VI). Part of the cerebellar cortex from adjacent sections is shown. cx—cerebral cortex; cb—cerebellum; hc—hippocampus; ht—hypothalamus; aon—anterior olfactory nucleus; vnl—ventral nucleus of lateral lemniscus; mtn—motor trigeminal nucleus; fn—facial nucleus; lrm—lateral reticular nucleus; CA1–CA3—hippocampus regions; DG—dentate gyrus; ml—molecular layer; gl—granule layer; egl—external granule layer; fl—fibrillar layer; pc—Purkinje cells.

According to the second model, the C-terminal domain is also divided on two similar parts, but the core sequence in each is $Cx_2Cx_4Hx_2C$, which is similar to the motif present in the RNA-binding proteins of retroviruses and retrotransposons (27). The main difference is the length of the H-C loop which is 4 amino acids long in retrovirus proteins and only 2 amino acids in neuro-d4 protein. But the most conservative C-H loops were quite similar (Fig.4c). In the latter model, the role of four Cx_2C sequences located at the distance of 11–21 amino acids upstream and downstream of each core structure is unclear. Structures like

‘superfingers’ could be proposed, but study of the protein is essential to resolve the organization of cysteine/histidine-rich region of the neuro-d4 gene product.

Widely spaced zinc-fingers have been found in several different proteins. In *Drosophila* genes *hunchback* (28), *Suvar(3)7* (29) and *teashirt* (6) and trypanosomal TRS-1 (30) clusters of similar CCHH fingers are spaced by 30–100 amino acids. In a human protein, CNRB, that binds to the sterol regulatory element (31), a retrovirus-like CCHC finger is separated from a tandem of the same six fingers by 34 amino acids. However, in proteins coded

by yeast RAD14 and homologous DNA-repair human and mouse genes (32) as well as in human RFP (33), RPT-1 (34) and PML (33, 34) spaced zinc-fingers belong to different types. Separation of finger structures within the *Suvar(3)7* protein molecule has been proposed to be connected with ability to bind large domains of DNA (29). In the neuro-d4 protein, two finger-like structures are separated by 55 amino acids. This is the first example of a combination of the *Krüppel*-type zinc-finger with the other finger-like structure in the same protein. The presence of this new type of cysteine/histidine-rich region together with a *Krüppel*-type zinc-finger, acidic region and nuclear localization signal strongly suggest that the neuro-d4 protein binds nucleic acids and is involved in the regulation of gene activity. Study of biological functions of different regions of recombinant neuro-d4 protein is now in progress.

All four differentially spliced neuro-d4 mRNA variants coded for different putative neuro-d4 proteins. As a result of a deletion of exon V (clone 9C), 30 amino acids, which includes the acidic domain (positions 152–161 in Fig. 2A) and the basic domain (positions 164–172), are absent in the protein encoded by this clone. It is interesting to note that some transcription factors (for example, the glucocorticoid receptor) have two nuclear translocation signals (37). The basic domain inside exon V could play the role of such an additional signal and its absence could change the behaviour of the resulting protein.

The additional 10 amino acids in the protein encoded by clone 7C change the structure of the cysteine/histidine rich region—extending the loop in the finger IV (first model, Fig. 4b) or the distance between two core sequences (second model). Such modification of presumptive nucleic acids binding domain might influence specificity of the protein. The same variant of differential splicing was found recently for human neuro-d4 gene (V.L.Buchman, unpublished).

Extension of neuro-d4 mRNA by the choice of a downstream donor site in exon I (clones 8C and 18C, Fig. 2 and 3) results in the appearance of multiple stop codons in all reading frames after the first ATG codon. In this case, a downstream ATG could serve as a initiation codon. For clone 18C, ATG encoding Met⁵⁶ (Fig. 3) could play such a role, producing a shorter protein but with all domains discussed above.

In clone 8C an alternative donor site is used also in exon VI. The resulting frameshift leads to interruption of the main reading frame by a stop codon in position 1548–1550 (Fig. 3). But another ATG codon (1457–1459 nt.) could function as a translation start which would return the translated protein to the main reading frame (Fig. 3). The resulting protein would lose 179 amino acids at the N-terminus, including the presumptive nuclear localization signal and *Krüppel*-type zinc-finger (the first Cys involving in coordination of Zn⁺⁺ is absent in this protein). This protein would, however, retain the C-terminal cysteine/histidine-rich region and the acidic region. The features and function of such a protein could be very different from the full-length neuro-d4 protein. The likelihood that both of the ATGs mentioned above are functional translation start sites is supported by the criteria of Kozak (19) and by modelling (38) of secondary structure of the different variants of neuro-d4 mRNA (A.Gulyaev, O.Reshetnikova and V.L.B., unpublished). A similar variant of differential splicing that causes a shift of the translation start site to a downstream initiation codon has been shown for the *Drosophila* gene *Krüppel* and is believed to play an important role in regulating *Krüppel* protein function in development (11). In human, another splicing variant of a neuro-

d4 transcript (deletion of exon VII) was found, that too leads to a loss of the *Krüppel*-type zinc-finger (V.L.Buchman, unpublished).

Expression of neuro-d4 is strictly restricted to the nervous system. Another example of a neurospecific gene encoding a zinc-finger protein is the mouse gene *mKr2*, however, substantial levels of *mKr2* mRNA were detected in the adrenal gland (12). Neuro-d4 mRNA cannot be detected in the adrenal gland by Northern hybridization. Neurospecific gene encoding the zinc-finger transcription factor NGFI-C belongs to a group of early response genes (39). In contrast, we failed to detect any change of neuro-d4 mRNA level in rat brain after metrazol-induced seizure (data not shown). Presumptive T-cell oncogene—*Tig* (40), or *11p15* (41), or rhombotin (42)—is the third known gene encoding a potential zinc-finger protein with expression restricted to the neuroendocrine lineage (43). It has been shown that this gene is expressed in postmitotic neurons, but perhaps not only in neurons (42). The patterns of the rhombotin transcription and splicing are complex but, in contrast to neuro-d4, presumptive proteins encoded by different mRNA are very similar (43).

By *in situ* hybridization we have shown that neuro-d4 mRNA-positive cells are neurons (see Results). Although, neuro-d4 transcripts were found in different regions of central and peripheral nervous system, they are more abundant in phylogenetically recent structures. The level of neuro-d4 mRNA is higher in developing than in mature central nervous system. This change in the level of neuro-d4 mRNA may reflect either different densities of neuro-d4 mRNA-positive neurons in various structures and periods or different levels of neuro-d4 expression in the neurons or both. For example, in adult rat brain, *in situ* hybridization shows that the intensity of staining of Purkinje cells and neurons in some brainstem nuclei is the same as of neurons in the hippocampus and cerebral cortex. On the other hand, during development of the cerebellum we have observed a switch in neuro-d4 expression from cells of the granule layers to the Purkinje cells. It will be particularly interesting to study the distribution of different neuro-d4 transcripts in nervous system during development and such experiments are in progress.

In conclusion, the complex, developmentally regulated pattern of neuro-d4 gene expression in nervous system suggests that it may play an important role in developing neurons. In the context of its possible role as a transcription factor, the presence of 'classic' *Krüppel*-type and new type of fingers in the same protein molecule, is of considerable interest.

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