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Evolutionary Studies of the Nerve Growth Factor Family Reveal a Novel Member Abundantly Expressed in Xenopus Ovary

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Summary

Evolutionary conservation of members of the NGF family in vertebrates was studied by DNA sequence analysis of PCR fragments for NGF, BDNF, and NT-3 from human, rat, chicken, viper, Xenopus, salmon, and ray. The results showed that the three factors are highly conserved from fishes to mammals. Phylogenetic trees reflecting the evolution and speciation of the members of the NGF family were constructed. In addition, the gene for a fourth member of the family, neurotrophin-4 (NT-4), was isolated from Xenopus and viper. The NT-4 gene encodes a precursor protein of 236 amino acids, which is processed into a 123 amino acid mature NT-4 protein with 50%–60% amino acid identity to NGF, BDNF, and NT-3. The NT-4 protein was shown to interact with the low affinity NGF receptor and elicited neurite outgrowth from explanted dorsal root ganglia with no and lower activity in sympathetic and nodose ganglia, respectively. Northern blot analysis of different tissues from Xenopus showed NT-4 mRNA only in ovary, where it was present at levels over 100-fold higher than those of NGF mRNA in heart.

Introduction

The nerve growth factor family includes β-nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin-3 (NT-3), also known as hippocampus-derived neurotrophic factor (HDNF). This family of proteins plays an important role in both the developing and the adult vertebrate nervous system, where they support neuronal survival (reviewed in Barde, 1989). Based on the amino acid sequence of the mouse NGF protein (Angeletti et al., 1973), DNA sequences coding for mouse and human NGF have been isolated (Scott et al., 1983; Ullrich et al., 1983). Comparison of mouse and human NGF showed that the protein is conserved within mammals, and in support of this, NGF-like activities have been isolated from several species (Harper and Thoenen, 1981). Subsequently, DNA sequences from bull (Meier et al., 1986), chick (Meier et al., 1986; Ebendal et al., 1986; Wion et al., 1986), cobra (Selby et al., 1987), rat (Whittemore et al., 1988), and guinea pig (Schwarz et al., 1989) NGFs were also determined. BDNF was first isolated from pig brain (Barde et al., 1982) and subsequently cloned as a cDNA from this tissue (Leibrock et al., 1989). The gene for NT-3 has been isolated from mouse (Hohn et al., 1990), rat (Maisonpierre et al., 1990; Ernfors et al., 1990a), and human (Rosenthal et al., 1990), using degenerate oligonucleotides based on the sequence similarity between the other two factors. The three factors show approximately 55% amino acid similarity to each other, and most sequence differences are present in five regions that contain amino acid motifs characteristic of each protein. The neurotrophic specificities in vitro of two of these proteins have recently been shown to be acquired by specific combinations of these variable regions (Ibáñez et al., 1991a).

NGF supports the development and maintenance of peripheral sympathetic and neural crest–derived sensory neurons (reviewed in Thoenen and Barde, 1980; Levi-Montalcini, 1987). No activity has been seen for BDNF in peripheral sympathetic neurons, but this factor supports in vivo the survival of both placode- and neural crest–derived sensory neurons (Hofer and Barde, 1988). The neurons sensitive to NT-3 in vivo remain to be identified. However, in explanted chick ganglia or dissociated neuronal cultures in vitro, the three factors support both overlapping and unique sets of neuronal populations, suggesting that NT-3 exerts both specific and overlapping neurotrophic activities also in vivo (Hohn et al., 1990; Maisonpierre et al., 1990; Ernfors et al., 1990a; Rosenthal et al., 1990). All three factors are expressed in specific sets of neurons in the brain, with the highest levels of mRNA for all three factors in the hippocampus (Ayer-LeLièvre et al., 1988b; Ernfors et al., 1990a, 1990b; Wetmore et al., 1991; Hofer et al., 1990; Phillips et al., 1990). In the brain, NGF has been shown to support basal forebrain cholinergic neurons (reviewed in Whittemore and Seiger, 1987; Thoenen et al., 1987; Ebendal, 1989a) and BDNF has been shown to stimulate the survival of these neurons in vitro (Alderson et al., 1990).

The effects of the three proteins are mediated by their interaction with specific receptors present on sensitive cells. Molecular clones have been isolated for the rat, human, and chicken NGF receptor (NGF-R), and nucleotide sequence analysis of these clones has shown that the NGF-R contains one plasma membrane–spanning domain, a cytoplasmic region, and an extracellular cysteine-rich amino-terminal domain (Johnson et al., 1986; Radeke et al., 1987; Large et al., 1989). The NGF-R shows a low, but significant, sequence similarity to the receptor for α-tumor necrosis factor (Schall et al., 1990) as well as to the lymphocyte surface antigens CD40 (Stamenkovic et al., 1989) and OX40 (Mallett et al., 1990). The NGF-R can occur in two apparent states, known as the low and high affinity states (Sutter et al., 1979; Landreth and Shooter, 1980; Schechter and Bothwell, 1981). The gene for the NGF-R appears to encode a protein that forms part of both the low and the high affinity states of the receptor (Hempstead et al., 1989), though only the high affinity

receptor has been proposed to mediate the biological activity of NGF. Both BDNF (Rodriguez-Tébar et al., 1990) and NT-3 (Ernfors et al., 1990a) can interact with the low affinity NGF-R, suggesting that the low affinity NGF-R may be, in an as yet unknown way, involved in mediating the biological effects of all three factors.

In the developing nervous system, NGF and its receptor have been shown to be synthesized in the target area and in the responsive neurons, respectively, at the time when the growing axon reaches its target (Davies et al., 1987). In agreement with this, the level of NGF mRNA in the developing chick embryo reaches a maximum at embryonic day 8 (E8) (Ebendal and Persson, 1988), which coincides with the time of sensory innervation. However, in the chick NGF-R mRNA is maximally expressed at early embryonic stages prior to neuronal innervation (Ernfors et al., 1988), and in the E3 chick embryo high levels of NGF-R mRNA have been detected in the mesenchyme, somites, and neural tube cells (Hallböök et al., 1990; Heuer et al., 1990a, 1990b). This observation, together with the fact that NGF mRNA is expressed in the E3 chick embryo at relatively high levels (Ebendal and Persson, 1988), indicates that NGF plays a role in early development that is distinct from its function as a neurotrophic factor. In agreement with this possibility, NGF has recently been shown to control proliferation and differentiation of E14 rat embryonic striatal precursor cells in culture (Cattaneo and McKay, 1990). In the chick embryo, BDNF and NT-3 mRNA are maximally expressed at E4.5 (Hallböök et al., submitted), and BDNF has been shown to control the differentiation of avian neural crest cells in vitro (Kalcheim and Gendreau, 1988).

Moreover, evidence for a nonneuronal function of NGF has also been presented. The still unexplained high levels of NGF found in the male mouse submandibular gland may indicate other functions for NGF (Levi-Montalcini, 1987). In the adult rat, NGF has been shown to induce DNA synthesis and to stimulate IgM secretion in B-cells (Otten et al., 1989). Furthermore, NGF mRNA is expressed in spermatocytes and early spermatids in the adult rat testis (Ayer-LeLièvre et al., 1988a), and the NGF protein is present in germ cells of all stages from spermatocytes to spermatozoa (Olson et al., 1987; Ayer-LeLièvre et al., 1988a). NGF-R mRNA has also been detected in the adult rat testis, where it is expressed in Sertoli cells under negative control of testosterone, and in the testis NGF has been suggested to control meiosis and spermiation (Persson et al., 1990).

The structural and functional similarities displayed by the members of the NGF family suggest that they originated from a common ancestral gene. This prompted us to carry out an evolutionary study of these factors in representative groups of vertebrates to obtain information that would allow a reconstruction of the evolutionary history of the distinct NGF-like genes. Our results show that the expansion of the NGF family preceded the vertebrate evolution and that the family includes a fourth member, neurotrophin-4 (NT-4). In line with the emerging view of both neurotrophic and nonneurotrophic roles for the members of the NGF family, the intriguing finding was made that in Xenopus the NT-4 gene is abundantly expressed in the ovary, implying a role for the NT-4 protein in oogenesis and/or early embryogenesis.

Results

Rationale for Study of the Evolutionary Conservation in the Nerve Growth Factor Gene Family

DNA fragments coding for NGF, BDNF, and NT-3 from human, rat, snake, frog, and fish were isolated using the polymerase chain reaction (PCR) technique with degenerate primers from conserved regions in these three proteins located between Lys-50 and Thr-56 for the upstream primer and between Try-99 and Asp-105 for the downstream primer (Figure 1A). The amplified region contains 3 of the 6 cysteine residues and covers approximately one-third of the mature molecules. A comparison of the amplified region in already characterized NGF molecules from different species shows that it contains two variable regions, Arg-59 to Ser-67 and Asp-93 to Ala-98. A hydrophilic stretch believed to be exposed on the surface of the molecule (Bradshaw, 1978), as well as the highly conserved regions Gly-68 to Try-76 and Thr-85 to Thr-91, is also included in the amplified region. The BDNF and NT-3 molecules have an extra amino acid between positions 94 and 95 of the mouse NGF protein, which is also included in the amplified region.

The sequences of the entire mature molecule of mouse NGF, BDNF, and NT-3 proteins were compared in order to calculate how representative the amplified region is of the complete molecule. The entire mature molecules show 65%/57% similarity (amino acid sequence similarity/nucleotide sequence identity) between NGF and BDNF, 70%/61% similarity between NGF and NT-3, and 68%/58% similarity between BDNF and NT-3. When comparing the region isolated in this study, the similarity between NGF and BDNF is 62%/53%, that between NGF and NT-3 is 67%/58%, and that between BDNF and NT-3 is 69%/60%. This strongly suggests that the region isolated in this study is representative of the entire molecule and that it can be used to monitor the evolutionary relationships among the different factors. Pairwise sequence comparisons were performed (Table 1) taking conservative amino acid replacements into consideration, using the comparison matrix of Schwartz and Dayhoff (1979). Therefore, comparisons of amino acid sequences given below and shown in Table 1 indicate percent similarity, not identity. Phylogenetic trees were constructed using parsimony analysis (Felsenstein, 1988; Swofford and Olsen, 1990). As shown below, all isolated DNA fragments with predicted amino acid sequences related to those of NGF, BDNF, and NT-3 contained conserved cysteine residues at the correct positions. This was used as an initial criterion for a sequence to be

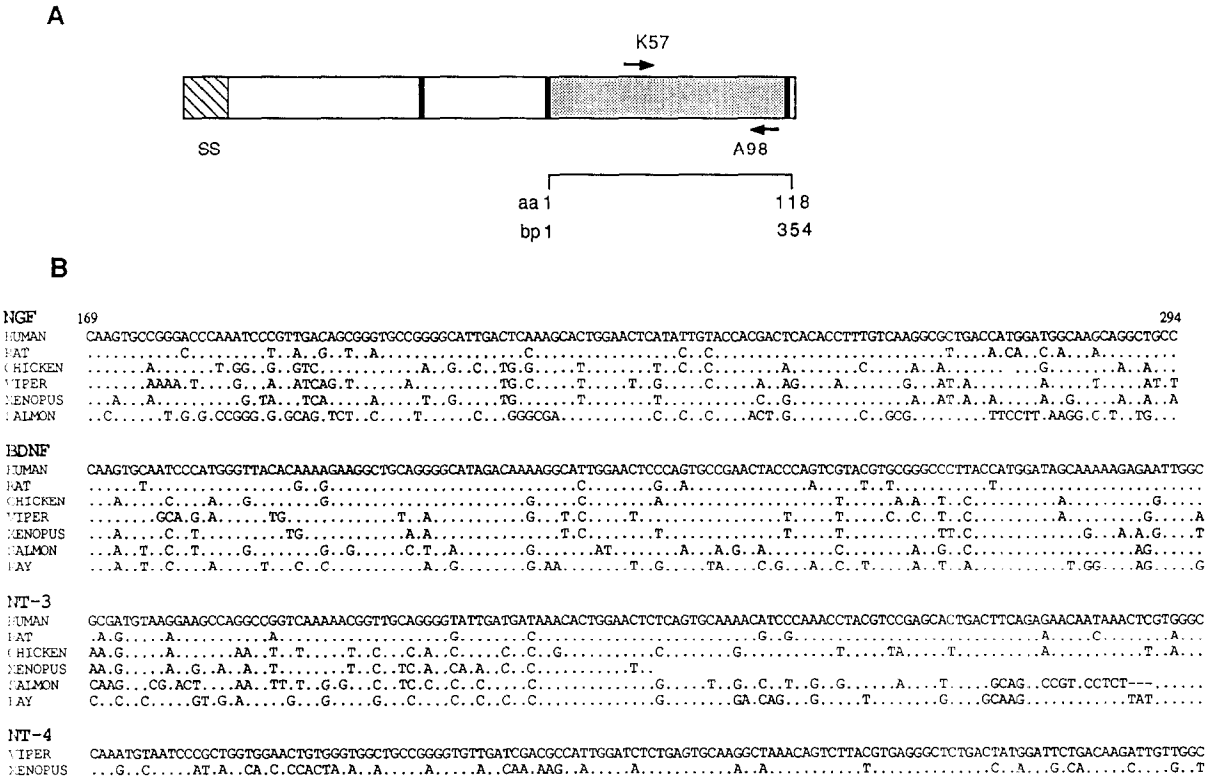

Figure 1. Alignments of DNA Sequences of the Isolated Fragments Coding for NGF, BDNF, NT-3, and the Novel Neurotrophic Factor NT-4 from Different Species

(A) Schematic representation of the mouse prepro-NGF molecule. The hatched box indicates the signal sequence (SS), black bars denotes proteolytic cleavage sites, and the shaded box represents the mature NGF. Regions used for the degenerate primers are indicated by arrows. The upstream primer is from the region coding for Lys-50 to Thr-56, and the downstream primer includes Try-99 to Asp-105. The amplified region comprises DNA sequences from basepair 168 to 294 in the mature NGF molecule, and in all members of the NGF family described so far, this region is located in one exon.

(B) Alignment of nucleotide sequences for NGF, BDNF, NT-3, and NT-4 isolated from different species. The fragments correspond to the coding region for amino acids 57 to 98 in the mature mouse NGF. Identical bases are indicated by dots. The numbering refers to nucleotides in the sequences of mouse mature NGF (Scott et al., 1983).

considered as a member of the nerve growth factor gene family.

NGF, BDNF, and NT-3 Are Highly Conserved during Evolution

NGF

The nucleotide sequence (Figure 1B) and the predicted amino acid sequence of the isolated fragments coding for NGF are highly conserved from fish to human (Figure 2). Most of the nonconservative amino acid changes were found in the variable regions Arg-59 to Ser-67 and Asp-93 to Ala-98 (Figure 2). The similarity between the Xenopus and human NGF sequences is 93%/79% (Table 1). Xenopus and chicken NGFs are

Table 1. Percent Nucleotide Identity and Amino Acid Similarity of NGF, BDNF, and NT-3

	NGF						BDNF							NT-3					
	Hum	Rat	Chi	Vip	Xen	Sal	Hum	Rat	Chi	Vip	Xen	Sal	Ray	Hum	Rat	Chi	Xen	Sal	Ray
Hum	-	95	93	86	93	69	-	100	95	86	95	100	93	-	100	100	100	81	88
Rat	87	-	88	81	88	69	91	-	95	86	95	100	93	91	-	100	100	81	88
Chi	80	73	-	90	100	74	88	84	-	91	91	95	93	81	83	-	98	81	86
Vip	73	72	75	-	90	67	83	78	85	-	88	86	84	X	X	X	X	X	X
Xen	79	73	83	76	-	74	73	82	85	82	-	95	88	73	79	86	-	86	90
Sal	60	61	60	51	53	-	82	80	83	75	78	-	93	67	64	71	71	-	74
Ray	X	X	X	X	X	X	77	76	78	74	75	78	-	76	72	71	72	71	-

Nucleotide identities and amino acid similarities were calculated with a VAX computer (software package from the UWGCG; Deveraux et al., 1984) according to the comparison matrix of Schwartz and Dayhoff (1979), taking conservative amino acid changes into consideration. The figures below the diagonals show percent nucleotide identity. The figures above the diagonals show the percent amino acid similarity. X indicates that the sequences were not isolated from those species (NGF from ray and NT-3 from viper). Hum, human; Chi, chicken; Vip, viper; Sal, salmon; Xen, Xenopus.

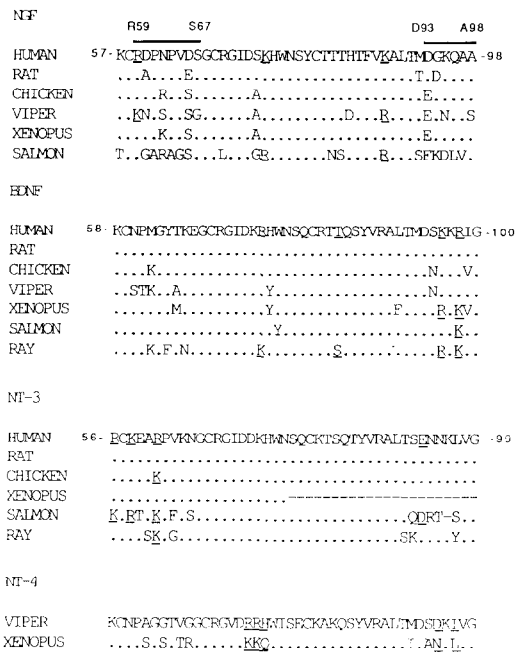

Figure 2. Alignment of Amino Acid Sequences Deduced for NGF, BDNF, NT-3, and NT-4 from Different Species

The numbering of the amino acids (single-letter code) is taken from the mature mouse NGF (Scott et al., 1983). Identical amino acids are indicated with dots. Positions that show conservative amino acid replacements in all species variants of the same factor are underlined. The broken line indicates that the corresponding sequence was not isolated. Bars represent variable regions in the different molecules (R59 to S67 and D93 to A98).

identical except for 1 conservative change from Lys-62 to Arg-62 (Figure 2). The sequences of viper and salmon NGF contain 11 and 19 amino acid differences (out of 42), respectively, compared with human NGF, whereas all other species only showed 4 differences. None of the NGF amino acid sequences isolated contained the extra amino acid residue present in both BDNF and NT-3 between Gly-94 and Lys-95 of the human NGF sequence. The interspecies relationships of the different NGF sequences were analyzed by the construction of a phylogenetic tree (Figure 3A). The salmon NGF sequence appears to have diverged more than the NGF sequences isolated from other species. No NGF sequence could be isolated from ray using the described PCR technique, suggesting that ray NGF sequences may be above the mismatch tolerance of the primers used in our PCR protocol. Alternatively, the absence of NGF in cartilaginous fishes would imply that NGF appeared after the splitting of the branch leading to the evolution of the bony fishes (some 450 million years ago) but before amphibians and higher vertebrates evolved from this branch (about 400 million years ago).

BDNF

DNA sequences similar to that of human BDNF were found in all species investigated (Figure 1B). The similarity in amino acid and nucleotide sequences between ray, the most primitive species investigated, and human is 93%/77% (Table 1). Only 2 nonconservative changes were seen outside the variable regions, whereas 10 similar changes were found in the two variable regions (Figure 2). In Xenopus, Leu-90 is replaced by a phenylalanine as a result of a single basepair mutation, C to T in the first position of the codon, and in salmon, Trp-77 is replaced by tyrosine as a result of a double mutation, changing the codon from TGG to TAT (Figure 1B). All isolated sequences contained an extra amino acid residue at position 96, compared with NGF (Figure 2). The BDNF sequences from different species appeared as a homogeneous group of sequences when analyzed by the parsimony method (Figure 3B).

NT-3

The nucleotide and predicted amino acid sequences for human, rat, chicken, Xenopus, salmon, and ray NT-3 are highly similar (Figure 1B; Figure 2). Most of the changes are silent mutations resulting from changes in the third position of the codons, usually transitions that preserve the pyrimidine or purine feature of the basepair. Only nonconservative amino acid changes were found within the two variable regions, and no amino acid replacements were seen outside the two variable regions. The salmon sequence lacks Asp-94, which is present in all other NT-3 molecules (Figure 2) and has a longer distance from the branching point in the phylogenetic tree than NT-3 sequences from other species (Figure 3C).

A Novel Member of the Nerve Growth Factor Gene Family: Neurotrophin-4

Additional DNA fragments were isolated from viper and Xenopus, and the predicted amino acid sequences revealed that they contained all 3 cysteine residues in the same positions as in NGF, BDNF, and NT-3 (Figure 1B; Figure 2). A comparison with the sequences of Xenopus NGF, BDNF, and NT-3 indicated that this new sequence is related, but not identical, to the sequences of the other members of the NGF family. The gene including this sequence was therefore named neurotrophin-4 or NT-4. Comparison of the nucleotide and amino acid sequences showed that Xenopus and viper NT-4 are 91%/73% similar. This similarity is in the same range as the similarity between Xenopus and viper NGF and BDNF (Table 1). As for the other members of the NGF family, nonconservative amino acid changes were seen only in the two variable regions (Figure 2).

Comparisons and Phylogeny of the Members in the Nerve Growth Factor Gene Family

A comparison of the phylogenetic trees for NGF, BDNF, and NT-3 showed longer branches in the NGF tree, indicating a higher rate of evolutionary change (Figures 3A–3C). The relationship of each member of the NGF family to the other members was studied by the construction of a phylogram comparing the deduced amino acid sequences for the four members

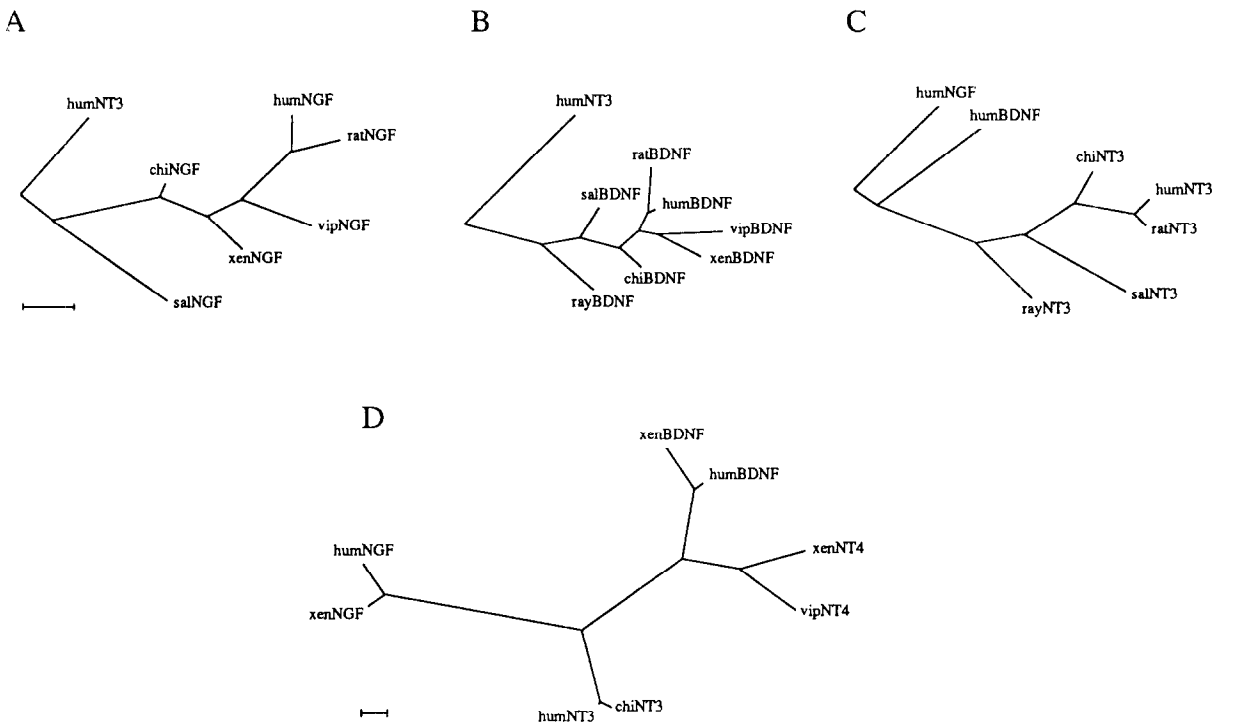

Figure 3. Deduced Phylogeny of Members of the NGF Family

Phylogenetic trees showing speciation of NGF (A), BDNF (B), and NT-3 (C) were constructed using analysis of nucleotide sequences. Human NT-3 was used as a reference point in (A) and (B); human NGF and human BDNF were used in (C). The scale bar in (A) represents a branch length corresponding to a relative difference score of 20. The same scale was used in (B) and (C). (D) shows a phylogram of the evolutionary relationship between the different members of the NGF family. The data were compiled from deduced amino acid sequences. The scale bar represents a branch length of 20. All trees shown are unrooted so that the branches are measured relative to one another with no outside reference. Abbreviations: chi, chicken; hum, human; sal, salmon; vip, viper; xen, Xenopus.

of the family. The phylogram showed that NGF is more closely related to NT-3 than to BDNF and NT-4 (Figure 3D). NT-3 is as related to NGF as to BDNF. NT-4 is clearly more related to BDNF than to the other two members.

Structural Features of the NT-4 Protein

To enable a more detailed characterization of the NT-4 gene and its gene product, we screened a Xenopus genomic library with the NT-4 PCR fragment and isolated a phage clone containing a 16 kb insert. From this insert, a 1.5 kb PstI fragment was subcloned and sequenced (Figure 4A). The nucleotide sequence contained an open reading frame encoding a 236 amino acid protein that showed several structural features characteristic of the other members of the NGF family. The amino terminus of the predicted NT-4 protein contains an 18 amino acid putative signal sequence in which a region of 4 amino acids is identical to the corresponding regions in pig and rat BDNF (Leibrock et al., 1989; Maisonpierre et al., 1990). A potential signal cleavage site, which is also identical to the one proposed for BDNF (Figure 4A), follows. A potential cleavage site for a 123 amino acid mature NT-4 protein is found after amino acid 113 in the prepro-NT-4 protein. A single predicted N-glycosylation site (Asn-Lys-Thr) is located 8 amino acids before the putative cleavage site.

A comparison of the mature NT-4 protein to the mature BDNF, NT-3, and NGF proteins from mouse revealed 60%, 58%, and 51% amino acid identity, respectively. Included in the mature NT-4 protein are all 6 cysteine residues involved in the formation of disulphide bridges (Figure 4B). The regions that are identical between NGF, BDNF, and NT-3 are also similar in the NT-4 protein. Most sequence differences between the NT-4 protein and the other three proteins were found within the same variable regions previously identified in the other members of the family.

Binding to the NGF-R and Neurotrophic Activity of NT-4

The 1.5 kb Xenopus PstI fragment was cloned in the expression vector pXM (Yang et al., 1986) and transiently expressed in COS cells. SDS-PAGE of conditioned media from transfected cells labeled with [^{35}S]cysteine showed an NT-4 protein with an M_r of 14K (Figure 5A). NGF protein produced and labeled in parallel dishes migrated somewhat faster than the NT-4 protein. This difference in mobility is most likely due to variations in the charge of the two proteins. Similar mobility differences have also been observed

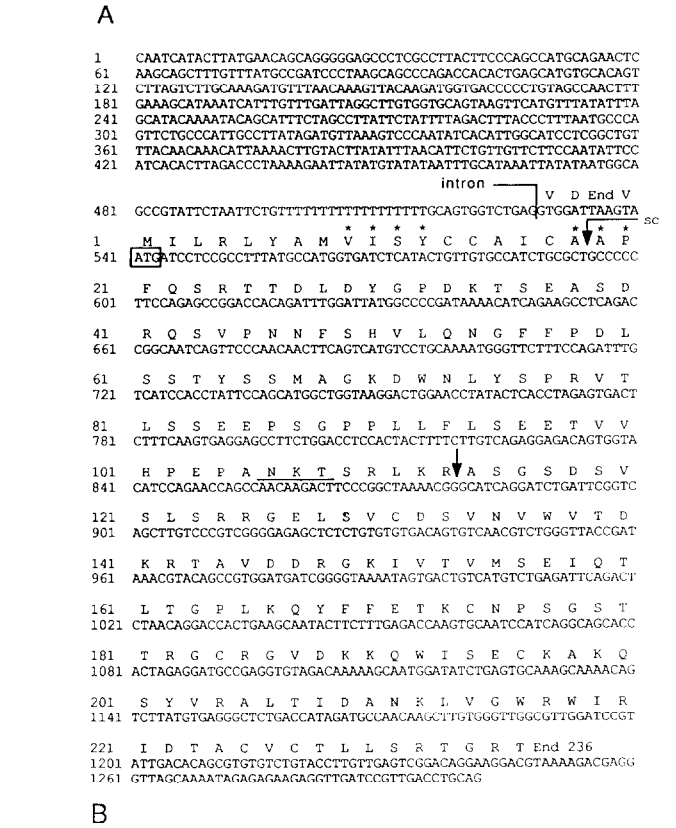

Figure 4. Sequence of Xenopus NT-4 and Comparison to NGF, BDNF, and NT-3

(A) A potential translation start site is boxed. A putative signal cleavage site is indicated by the arrow labeled SC. Amino acids within the signal sequence that are identical between Xenopus NT-4 and pig and rat BDNF are indicated with stars. A consensus sequence for N-glycosylation is underlined, and the arrow indicates the presumptive start of the mature NT-4 protein.

(B) Amino acid (single-letter code) sequence comparison of Xenopus NT-4 with mouse NGF (Scott et al., 1983), mouse BDNF (Hofer et al., 1990), and mouse NT-3 (Hohn et al., 1990). Identical amino acid replacements compared with the NT-4 amino acid sequence are shown by dots. Sequences that differ between NGF, BDNF, and NT-3 also differ in the sequence of the NT-4 protein.

for NGF proteins with identical sizes from different species (Ibáñez et al., 1991b).

Conditioned media from transfected COS cells containing equal amounts of rat NGF and Xenopus NT-4 protein were tested for their ability to compete for binding of ^{125}I-labeled NGF to its receptor on PC12 cells. Binding assays were done at 37°C and under conditions in which 80% of the ^{125}I-NGF associated to the cells is bound to the low affinity NGF-R (Sutter et al., 1979). Similar concentrations of NGF and NT-4 (6×10^{-10} M) were required to displace 50% of the ^{125}I-NGF from the PC12 cells, indicating that the two proteins bind to the low affinity NGF-R with a similar affinity (Figure 5B). At higher concentrations, the NT-4 protein was less efficient in displacing ^{125}I-NGF, suggesting that in this case the remaining ^{125}I-NGF associated with the cells was bound to high affinity or internalized receptors. The fact that this difference could not be seen in a parallel assay performed at 0°C (data not shown), in which no membrane mobilization or internalization occurs, suggests that the NT-4 protein is not able to compete with NGF for internalization, a process known to be mediated exclusively through the high affinity receptors (Olender and Stach, 1980; Bernd and Greene, 1984; Hosang and Shooter, 1987).

The NT-4 protein transiently expressed in COS cells was tested for its ability to promote neurite outgrowth from explanted embryonic chick ganglia. A clear stimulation of neurite outgrowth from explanted chicken dorsal root ganglia was seen (Figure 6A). Comparison of dose–response curves using equal amounts of NT-4 and NGF protein revealed that the activity obtained with NT-4 was lower than that seen with NGF (Figures 6A and 6B). Recombinant NT-4 and BDNF proteins stimulated neurite outgrowth in the dorsal root ganglia to a similar extent (Figures 6A and 6C). The NT-4 protein elicited a weak, but consistent, neurite outgrowth from the nodose ganglia (Figure 6G), whereas no activity could be detected in sympathetic ganglia (Figure 6E). This is in contrast to NGF, which markedly stimulates neurite outgrowth from sympathetic ganglia (Figure 6F), and NT-3, which showed a clear activity in the nodose ganglia (Figure 6H). As for NT-4, the neurite outgrowth-promoting activity of BDNF in the nodose ganglia (Figure 6I) was lower than the activity seen with NT-3.

Expression of NT-4 mRNA in Different Xenopus Tissues

Polyadenylated RNA was prepared from 11 different Xenopus tissues and used for Northern blot analysis. Hybridization with the Xenopus NT-4 probe revealed high levels of two NT-4 transcripts of 2.3 kb and 6.0 kb in the ovary (Figure 7A). In contrast, the level of NT-4 mRNA was below the detection limit in all other tissues analyzed. Hybridization with a Xenopus NGF probe showed a 1.3 kb NGF mRNA in the heart (Figure 7A) and brain (data not shown). However, the amount of NGF mRNA in these tissues was on the order of 100 times lower than the level of NT-4 mRNA in the ovary. NGF mRNA was also detected in the ovary, though the amount of NGF mRNA was approximately 100 times lower than the level of NT-4 mRNA in this tissue (Figure 7B). The levels of BDNF and NT-3 mRNAs in ovary were both below the detection limit (Figure 7B).

Discussion

Evolutionary Conservation of the Nerve Growth Factor Gene Family

We have used the PCR in combination with degener-

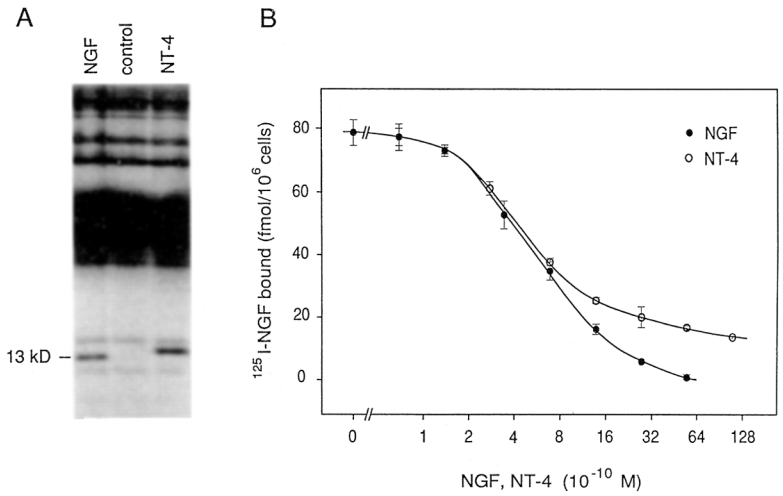

Figure 5. Transient Expression of the Xenopus NT-4 Protein in COS Cells and Its Interaction with NGF-Rs on PC12 Cells

(A) SDS–PAGE of conditioned media from in vivo labeled COS cell cultures (3×10^5 cpm loaded in each lane) transfected with the rat NGF gene, a control plasmid without insert, or the Xenopus NT-4 gene. Shown is an autoradiograph of the dried gel after an overnight exposure to X-ray film.

(B) Serial dilutions of transfected COS cell medium containing equal amounts of NT-4 (open circles) or NGF (closed circles) protein were assayed for their ability to displace ^{125}I-NGF from its receptor on PC12 cells. Binding assays were performed at 37°C using 1.5×10^{-9} M ^{125}I-NGF and 1×10^6 cells per ml. Medium from mock-transfected cells failed to displace binding of ^{125}I-NGF from PC12 cells. Each point represents the mean ± SD of triplicate determinations.

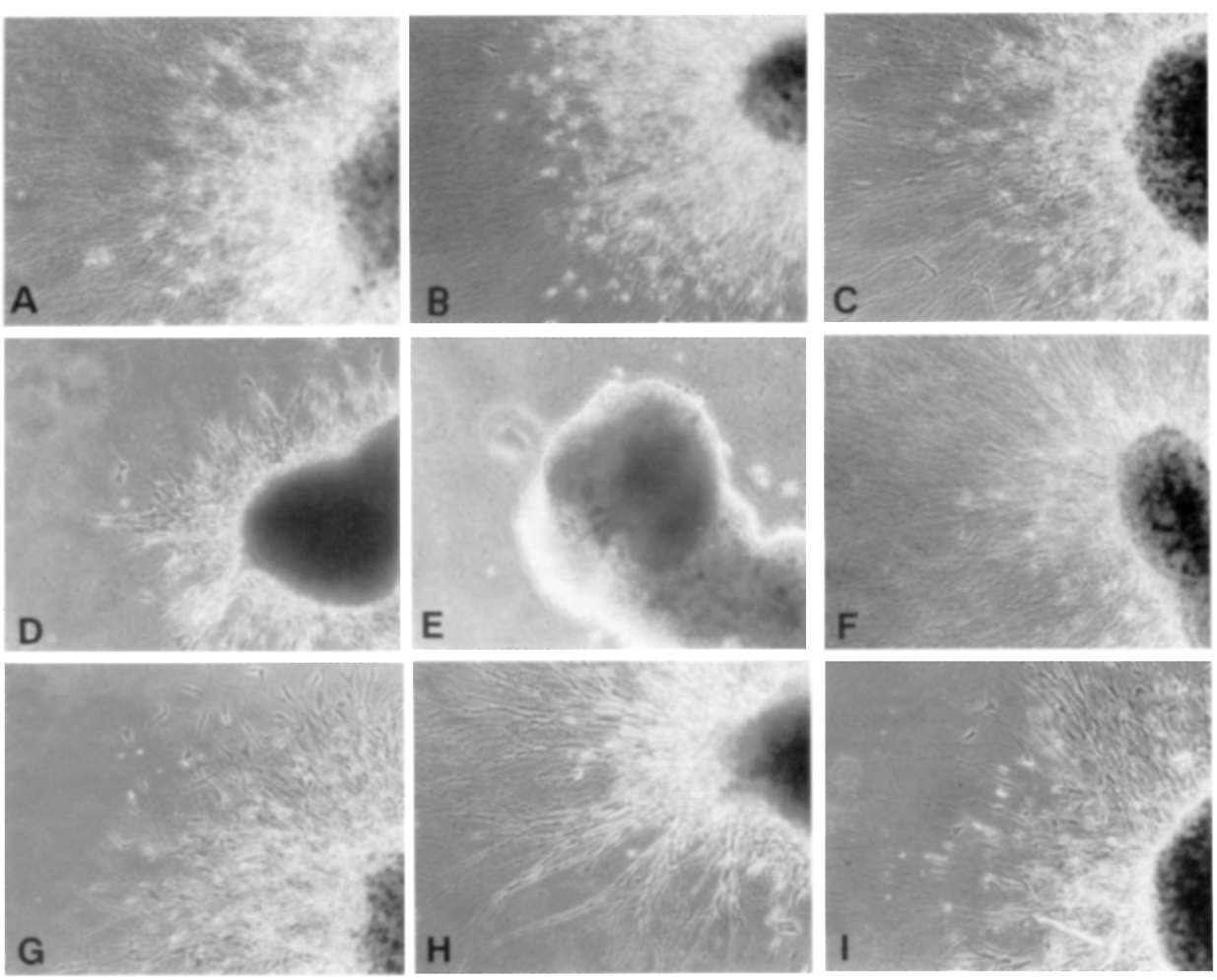

Figure 6. Stimulation of Neurite Outgrowth from Chicken Embryonic Ganglia

(A, B, and C) Neurite outgrowth elicited in dorsal root ganglia with recombinant NT-4 protein (A), recombinant NGF (B), and BDNF protein (C). (D) The response of dorsal root ganglia to conditioned medium from mock-transfected cells. (E and F) Stimulation of neurite outgrowth from sympathetic ganglia in response to NT-4 (E) or NGF (F). (G, H, and I) Nodose ganglia stimulated with recombinant NT-4 (G), NT-3 (H), and BDNF (I) proteins. All figures are bright-field micrographs of ganglia after 1.5 days in culture.

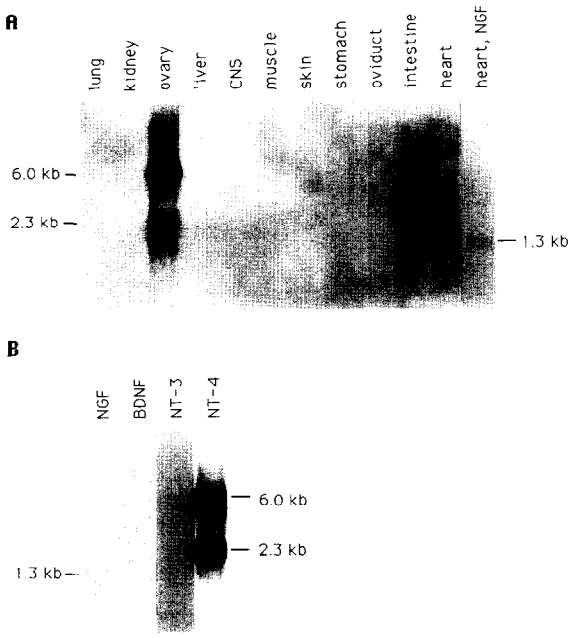

Figure 7. Detection of NT-4 mRNA in Different Xenopus Tissues

(A) Poly(A)$^+$ RNA (10 μg per slot) from the indicated tissues of adult female Xenopus was electrophoresed in a formaldehyde-containing agarose gel, blotted onto a nitrocellulose filter, and hybridized to a 600 bp HincII fragment from the 3′ exon of the Xenopus NT-4 gene. For comparison, the filter was also hybridized to a 180 bp PCR fragment from the Xenopus NGF gene (lane marked heart, NGF). The filter hybridized to the NT-4 probe was exposed for 2 days; the filter hybridized to the NGF probe was exposed for 2 weeks. A prolonged, 2 week exposure of the filter hybridized to the NT-4 probe did not reveal NT-4 mRNA in any tissues other than the ovary, which includes oocytes of different stages. The lane labeled CNS includes brain and spinal cord.

(B) Poly(A)$^+$ RNA (10 μg) from Xenopus ovary was analyzed for the expression of the four members of the NGF family. Each filter was hybridized with the indicated probes obtained by labeling of PCR fragments from their respective Xenopus genes. The location of the labeled PCR fragments in the 3′ exon of their genes is shown in Figure 1A. The filters were washed at high stringency and exposed to X-ray films for 5 days.

ate oligonucleotide primers to isolate the genes for different members in the NGF family from different species. A comparison of the nucleotide and amino acid sequences of the entire mature mouse NGF, BDNF, and NT-3 proteins revealed similarities that are the same as those obtained by comparing the region of the genes analyzed in this study. Hence, this region appears to be representative of the rest of the gene and can therefore be used to study the evolutionary conservation of the entire mature protein.

The NGF, BDNF, and NT-3 genes from different species include regions that show complete identity between fishes and mammals, as well as regions with lower similarity. A comparison of NGF sequences from different species with the corresponding sequences of BDNF or NT-3 showed that the NGF gene is less conserved in vertebrates than both BDNF and NT-3. The two latter genes appear to be equally conserved in all species studied, except in salmon, in which NT-3 is less conserved than BDNF. In this context, it is interesting to speculate about the fact that the molecular clock seems sped up in some branches, notably NGF, and not in others. It is generally believed that there is a selective force that preserves the correct tertiary structure of a protein (Dickerson, 1971; Kimura and Ohta, 1973). The difference in the evolutionary conservation of the three factors suggests that there has been a higher selective pressure on BDNF and NT-3 than on the NGF gene. Environmental changes have been proposed to lead to changes in the selective pressure altering the performance optimum of a specific gene product (Kimura, 1983). In this context, it is possible that the more extensive evolutionary changes seen in NGF compared with BDNF and NT-3 reflect the fact that the function of NGF has changed more during evolution. Structure–function studies of NGF have shown that this molecule can tolerate considerable structural changes without loss or modification of its activity profile (Ibáñez et al., 1990, 1991a), suggesting that the lower degree of evolutionary conservation of NGF could be due to a more stable structure of this protein, which is therefore less easily perturbed by substitutions. Another possible explanation is that the regions of the genome where the genes for the different factors are located have different general mutation rates. Different mutation rates have been shown for noncoding regions of the genome (Wolfe et al., 1989), but it is less clear whether this can lead to an increased number of changes in coding regions.

Salmon NGF and NT-3 are notably more different when compared with these molecules in other species. Some amino acids, including the Thr-82 and the His-Thr-Phe at positions 85 to 87 in NGF, as well as the absence of the amino acid between positions 94 and 95 (compared with the two other proteins), are consistent features of the NGF protein. The fact that the isolated salmon sequence contains all of these NGF-specific motifs argues that it is not an additional member of the family, but rather represents salmon NGF. In contrast to all other NT-3 sequences studied, salmon NT-3 lacks the amino acid in position 95. Since the extra amino acid is present in ray NT-3, it is likely that the common ancestor of ray and salmon had an ancestral NT-3 sequence which included the extra amino acid in position 95. Therefore, the changes in the salmon NT-3 molecule must have occurred after this gene split from the common ancestor. Most of the changes in the amino acids of the salmon sequence are in the same regions that vary, to a lesser degree, also in the other species, strongly suggesting that the isolated salmon NGF or NT-3 sequences are not pseudogenes. The greater divergence of salmon NGF and NT-3, compared with the other species, probably reflects the high degree of evolutionary expansion of the bony fishes (Young, 1981).

The results in this study indicate that the NGF family probably already existed 500 million years ago in the

primitive fishes, which were the ancestors of todays higher vertebrates. The gene family could have been formed by gene duplication, which is believed to be the most common mechanism whereby new genes evolve (Li, 1983). Duplications of functional genes could have been facilitated, since all information required for the synthesis of a biologically active protein is contained within a 3′ exon (Hallböök et al., 1988; Leibrock et al., 1989; Hohn et al., 1990). The formation of the family has involved several gene duplications (Figure 3D). Since NT-4 is more closely related to BDNF than to NT-3 or NGF, it appears that NT-4 and BDNF were formed from a common ancestral gene. However, since no progenitor-like molecule for all four factors can be distinguished from the present data, the evolutionary relation of the putative BDNF/NT-4 ancestor to the ancestors of NGF and NT-3 cannot be established. The topology of the phylograms using data from different species is in general agreement with the consensus evolutionary relationship among different species (Young, 1981). However, for both NGF and BDNF, the chicken sequences show an earlier branching in the phylogram than expected. Comparison of NT-4, NGF, and BDNF from viper and Xenopus revealed that the NT-4 sequences in these species have 11 amino acid replacements, compared with 9 and 8 replacements in NGF and BDNF, respectively. This suggests that in these species, NT-4 has diverged with a rate that is comparable to, or faster than, the rate of NGF or BDNF divergence.

Replacements of highly conserved amino acids in the NGF molecule do not abolish the biological activity, but in many cases these affect the amount of protein produced (Ibáñez et al., 1990), indicating that there are constraints other than the biological activity, such as protein stability, which may be important for the conservation of the NGF protein. In addition, the fact that all members of the NGF family can interact with the low affinity NGF-R suggests that the complete conservation of certain regions in these factors may be due to constraints on these genes to retain proteins that can interact with the NGF-R. The basic mechanisms and strategies for the early ontogeny of the embryo are similar in all vertebrates and presumably involve genes that are conserved in all vertebrates (Bonner, 1982). The evolutionary conservation of the neurotrophic factors is therefore consistent with the notion that they are important in early embryonic development in many different species.

The hippocampus contains the highest levels of NGF, BDNF, and NT-3 mRNA in the brain (Ernfors et al., 1990b). It is a highly specialized structure derived from the archipallium, which first appeared in the brains of amphibians and reptiles (Young, 1981; Romer, 1976). The mammalian hippocampus is important for memory, learning, and cognitive functions known to be associated with high neuronal plasticity (Crutcher and Collins, 1982). These demands may have generated a selective pressure during phylogeny for plasticity-promoting mechanisms, possibly mediated by neurotrophic factors. However, the results in this study clearly show that the duplication event of the genes for the neurotrophic factors preceded by far the formation of the hippocampus. This finding indicates that the neurotrophic factors did not evolve as a consequence of the formation of the hippocampus and supports the notion that the neuronal plasticity in this brain region is at least in part due to these molecules.

The organization of the nervous system of primitive vertebrates, i.e., cartilaginous fishes, shows some basic similarities to the nervous system of higher vertebrates. The cranial nerves and the somatic sensory and autonomic nervous systems in cartilaginous fishes are in general similar to those of higher vertebrates (Young, 1981). It is therefore likely that the principles of neurotrophic interactions are the same in both primitive and higher vertebrates. The evolutionary conservation of the NGF-like neurotrophic factors also in primitive vertebrates suggests that these factors first evolved in invertebrates and were later adapted to function in the development of the vertebrate nervous system.

Structural Features and Neurotrophic Activities of NT-4

Our study of the evolutionary conservation of the NGF family led to the isolation of a novel member of this family, named neurotrophin-4 or NT-4. PCR fragments from the NT-4 gene were isolated from Xenopus and viper, and a genomic clone was subsequently isolated from Xenopus. Nucleotide sequence analysis of this clone revealed an open reading frame for a 236 amino acid protein, which showed several structural features resembling those of the three other members of the NGF family. These include the presence of a putative amino-terminal signal sequence and a potential N-glycosylation site close to a proteolytic cleavage site that predicts a 123 amino acid mature NT-4 protein. The size of the mature NT-4 protein is 4 amino acids longer than that of BDNF and NT-3 and 5 amino acids longer than the mature NGF protein. Within the mature NT-4 protein, all 6 cysteine residues involved in the formation of disulphide bridges are conserved. The NT-4 protein differs from the other members of the family in the same regions that vary among the sequences of the three other family members. As for NGF, BDNF, and NT-3, the entire prepro-NT-4 protein is encoded in one single exon. Hence, both the gene organization and the structural features of the predicted protein strongly argue that the NT-4 gene is an additional member of the NGF family. The fact that the NT-4 gene was isolated from both reptiles and amphibians suggests that it is present in several different species, though this has to be confirmed by isolating the NT-4 gene from mammals.

Both BDNF and NT-3 have been shown to interact with the low affinity NGF-R (Rodriguez-Tébar et al., 1990; Ernfors et al., 1990a). The Xenopus NT-4 protein displaced ^{125}I-NGF from its low affinity receptor on

PC12 cells, indicating that the fourth member of this family can also interact with the low affinity NGF-R. The comparison of displacement curves obtained at 37°C and 0°C suggests that the NT-4 protein cannot compete for binding to the high affinity NGF-R. The protein encoded by the low affinity NGF-R gene appears to form part of both the low and the high affinity receptors (Hempstead et al., 1989). The mechanism by which two kinetically different receptors are formed from the same receptor gene is not known, although it has been proposed that the two states can be generated by the formation of a complex between the cytoplasmic domain of the receptor and an intracellular protein (Radeke et al., 1987; Meakin and Shooter, 1991). Alternatively, a high affinity receptor chain may be encoded by a separate gene and, similar to the interleukin-2 receptor (Hatakeyama et al., 1989) and the platelet-derived growth factor receptor (Matsui et al., 1989), the two receptor chains may form a dimer that constitutes the high affinity receptor. The fact that all four members of the NGF family can interact with the low affinity NGF-R suggests that the low affinity state of the NGF-R may be, in an as yet unknown way, involved in mediating the biological effects of all these factors. In this context, it is interesting to note that the low affinity NGF-R gene has been shown to be expressed in many tissues of both neuronal and nonneuronal origin not known to respond to NGF. These include mesenchyme, somites, and neural tube cells in the early chick embryo (Hallböök et al., 1990; Heuer et al., 1990a, 1990b), as well as developing and regenerating spinal cord motoneurons (Ernfors et al., 1989, 1991). It would therefore be of interest to investigate whether the NT-4 protein is of functional importance in any of these tissues or neuronal populations.

The neurotrophic activity of the NT-4 protein was assayed on explanted chick embryonic ganglia, and as for the other three members of the NGF family, the NT-4 protein showed a clear stimulation of neurite outgrowth from dorsal root ganglia. However, when compared with NGF, the NT-4 protein showed lower activity in dorsal root ganglia. Both BDNF and NT-3 readily elicit neurite outgrowth in explanted nodose ganglia, though the response with NT-3 was consistently stronger than that with BDNF. NGF strongly stimulates neurite outgrowth in sympathetic ganglia, and NT-3 also has activity in this ganglia, though it is much lower than that of NGF (Maisonpierre et al., 1990; Ernfors et al., 1990a; Ibáñez et al., 1991a). NT-4 showed weaker activity in nodose ganglia compared with NT-3 and no activity in the sympathetic ganglia. The spectrum of the biological activity of NT-4 on peripheral explanted ganglia resembles that of BDNF, which is in agreement with the fact that NT-4 is structurally similar to BDNF.

The NT-4 Gene Is Abundantly Expressed in Xenopus Ovary

Northern blot analysis of 11 different tissues from Xenopus showed high levels of NT-4 mRNA in the ovary, whereas the level of NT-4 mRNA was below the detection limit in all other tissues examined. Two NT-4 mRNAs of 2.3 kb and 6.0 kb were seen in the oocytes. The presence of two transcripts from the same gene has previously been observed for BDNF, in which case two mRNAs of 1.4 kb and 4.0 kb are present in the rat brain (Leibrock et al., 1989; Maisonpierre et al., 1990; Ernfors et al., 1990a). Hybridization to a Xenopus NGF probe revealed NGF mRNA in the Xenopus heart, most likely as a result of NGF mRNA expression in target tissues for neuronal innervation. The level of NGF mRNA in the heart was, however, more than 100-fold lower than the level of NT-4 mRNA in the ovary. Since the high level of NT-4 mRNA in the ovary does not correlate with neuronal innervation, it appears unlikely that the NT-4 protein has only a neurotrophic function in this case. Instead, the abundant expression of NT-4 mRNA in Xenopus ovary implies an additional and important nonneurotrophic function for the NT-4 protein. NGF mRNA was also detected in Xenopus ovary though at almost 100 times lower levels than those of NT-4 mRNA; BDNF and NT-3 mRNAs were not detected in this tissue.

mRNAs for two growth factors have been described as maternal mRNAs in Xenopus oocytes. One of these mRNAs encodes a protein with strong similarity to basic fibroblast growth factor (Kimelman and Kirschner, 1987); the other mRNA encodes a protein homologous to transforming growth factor α (Weeks and Melton, 1987). These factors have been suggested to function as morphogens for the formation of mesoderm and the subsequent induction of this tissue into the neural tube. In the rat, in situ hybridization studies have revealed NT-3 mRNA in the epithelium of secondary and tertiary follicles, and a role for NT-3 in oogenesis has been suggested (Ernfors et al., 1990b). The abundant expression of NT-4 mRNA in Xenopus ovary, including the oocytes, implies that the NT-4 protein could play a role in oogenesis and/or early embryogenesis. Additional studies on the cellular localization of NT-4 mRNA in the ovary should help to clarify the function of the NT-4 protein in this tissue.

Experimental Procedures

DNA Preparation

Genomic DNA was isolated by standard procedures (Davis et al., 1986) from human leukocytes and from liver of Sprague-Dawley rat, frog (Xenopus laevis), and ray (Raja clavata). Genomic DNA was also obtained from salmon (Salmon) and from the elephant snake (Vipera lebetina). The DNA was precipitated with ethanol, collected using a glass hook, washed in 80% ethanol, dried, and dissolved in water to a final concentration of 1 mg/ml. Salmon DNA (Sigma, St. Louis, MO) was dissolved in water, extracted twice with phenol and once with chloroform, and precipitated with ethanol.

PCR, Molecular Cloning, and DNA Sequencing

Six separate mixtures of 28-mer oligonucleotides representing all possible codons corresponding to the amino acid sequence KQYFYET (5′ oligonucleotide) and WRFIRID (3′ oligonucleotide) (Figure 1A) were synthesized on an Applied Biosystem A381 DNA synthesizer. The 5′ oligonucleotide contained a synthetic EcoRI site, and the 3′ oligonucleotide contained a synthetic HindIII

site (Knoth et al., 1988; Nunberg et al., 1989). Each mixture of oligonucleotides was then used to prime the amplification of 0.8 μg of genomic DNA using the PCR (Taq DNA polymerase; Promega) (Saiki et al., 1985). The PCR products were restricted with HindIII and EcoRI, analyzed on a 2% agarose gel, and cloned into plasmid Bluescript KS+ (Stratagene, La Jolla, CA). The size of the amplified region plus primers is 179 bp for NGF and 182 bp for BDNF and NT-3. As a result of internal EcoRI sites in some cases, shorter fragments of 144 bp and 95 bp were also isolated. The cloned DNA fragments were sequenced using the dideoxy nucleotide chain termination method (Sanger et al., 1977) with T7 DNA polymerase (Pharmacia, Uppsala). Between 2 and 20 independent clones were sequenced for each gene and species, and altogether more than 200 independent clones were sequenced.

Approximately 2,000,000 clones from a Xenopus genomic library prepared by insertion of MboI-digested genomic DNA in the BamHI site of phage λ EMBL-3 were screened using conventional procedures with a 182 bp PCR fragment of Xenopus NT-4 labeled with [α-^{32}P]dCTP by nick translation to a specific activity of approximately 5 × 10^8 cpm/μg. Hybridization was carried out in 4× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate [pH 7.0]), 40% formamide, 1× Denhardt solution, 10% dextran sulfate at 42°C. The filters were washed at 55°C in 0.1× SSC, 0.1% SDS and exposed to Kodak XAR-5 films at −70°C. Eight phage clones were isolated, and a hybridizing 1.5 kb PstI fragment from one of these clones was subcloned in the plasmid pBS-KS (Stratagene). The nucleotide sequence of the subcloned fragment was determined by the dideoxy chain termination method (Sanger et al., 1977).

Computer Analysis of the Sequence Data

DNA and amino acid sequence comparisons and alignments shown in Table 1 were performed on a VAX computer using UWGCG softwear (Devereux et al., 1984). The results of comparing amino acid sequences using the UWGCG programs are presented as percent amino acid similarity or nucleotide identity between the sequences, taking conservative amino acid changes into consideration (Gribskov and Burgess, 1986; Schwartz and Dayhoff, 1979). Phylogenetic Analysis Using Parsimony (PAUP version 3.0f) was used for the construction of the phylograms (Felsenstein, 1988; Swofford and Olsen, 1990). Searches for the most probable trees were run using both exhaustive and heuristic (branch swapping) algorithms.

Production of Recombinant Protein, Binding Assay to PC12 Cells, and Assays of Neurotrophic Activities

For transient expression of recombinant proteins in COS cells, appropriate DNA fragments were cloned in the vector pXM (Yang et al., 1986). For NT-4 the sequenced 1.5 kb PstI fragment from Xenopus was cloned in pXM, and for NGF a 771 bp BstEII–PstI fragment from the 3′ exon of the rat NGF gene was used (Hallböök et al., 1988). To express BDNF protein, a PCR-amplified fragment containing the prepro-BDNF coding sequence from the mouse BDNF gene (Hofer et al., 1990) was also subcloned in pXM. For NT-3, a 1020 bp rat cDNA clone was inserted in pXM (Ernfors et al., 1990a).

COS cells (Gluzman, 1981) grown to about 70% confluency were transfected with 25 μg of plasmid DNA per 100 mm dish using the DEAE-dextran–chloroquine protocol (Luthman and Magnusson, 1983). Transfected cells were then grown in complete medium (DMEM plus 10% FCS), and conditioned medium was collected 3 days after transfection. Dishes (35 mm) transfected in parallel were grown over the third night after transfection in the presence of 200 μCi/ml [^{35}S]cysteine (Amersham, UK). Aliquots (10–20 μl each) of the in vivo labeled conditioned media were analyzed by SDS–PAGE in 13% polyacrylamide gels. The gels were treated with EnHance (New England Nuclear, Boston, MA), dried, and exposed to Kodak XAR5 films with intensifying screens for 24–48 hr at −80°C. Autoradiographs were scanned in a Shimadzu densitometer, and the relative amounts of the different recombinant proteins were estimated by calculating the area corresponding to each protein relative to that obtained with rat NGF. The absolute amount of rat NGF protein was assessed by quantitative immunoblotting of conditioned media using standards of purified mouse NGF (Ibáñez et al., 1991b) and was used to determine the protein concentration in the samples containing the other recombinant proteins.

For binding assay of recombinant proteins to PC12 cells (Greene and Tischler, 1976), mouse NGF was labeled with ^{125}I by the chloramine-T method to an average activity of 7 × 10^7 cpm/μg. Steady-state binding was measured in competition assays performed at 37°C or 0°C using 1 × 10^6 cells per ml, 1.5 × 10^{-9} M ^{125}I-NGF, and serial dilutions of conditioned media containing equivalent amounts of NGF or NT-4. All components were added at the same time, and cells were collected by centrifugation after equilibrium was reached (1–2 hr incubation). Control experiments using medium from mock-transfected COS cells showed that other proteins present in the conditioned medium had no effect on the binding of ^{125}I-NGF to PC12 cells. Nonspecific binding was measured in a parallel incubation to which at least a 1000-fold excess of unlabeled NGF was added. All results were corrected for this nonspecific binding, which was always less than 10% of the total binding.

The biological activities of the different proteins were measured by the ability of transfected COS cell conditioned media, containing equal amounts of recombinant protein, to stimulate neurite outgrowth from explanted sympathetic, nodose, and dorsal root ganglia from E9 chicken embryos (Ebendal, 1984, 1989b). Serial dilutions of conditioned medium were assayed, and the fiber outgrowth was scored.

RNA Preparations and Blot Analysis

The indicated tissues from adult female Xenopus were dissected and frozen in liquid nitrogen. The brain and spinal cord were pooled. Several lobes of the ovary were dissected out, including oocytes of different stages. The frozen tissue samples were homogenized in 4 M guanidine isothiocyanate, 0.1 M β-mercaptoethanol, 0.025 M sodium citrate (pH 7.0) and homogenized three times for 15 s with a Polytron. Each homogenate was layered over a 4 ml cushion of 5.7 M CsCl in 0.025 M sodium citrate (pH 5.5) and centrifuged at 15°C in a Beckman SW41 rotor at 35,000 rpm for 16 hr (Chirgwin et al., 1979). Poly(A)$^+$ RNA was purified by oligo(dT)–cellulose chromatography (Aviv and Leder, 1972), and the recovery of RNA was quantified spectrophotometrically before use in RNA blot analysis. Poly(A)$^+$ RNA (10 μg) from each sample was electrophoresed in a 1% agarose gel containing 0.7% formaldehyde. UV-transillumination of the stained gel was used to confirm that all samples contained similar amounts of intact RNA. The gel was then transferred to a nitrocellulose filter. The filter was hybridized to the indicated DNA probes. The probes were labeled with [α-^{32}P]dCTP by nick translation to a specific activity of around 5 × 10^8 cpm/μg, and the hybridization was carried out as described above. Filters were washed at high stringency (0.1 × SSC, 0.1% SDS, 54°C) and exposed to Kodak XAR-5 films.

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