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Mammalian neurotrophin-4: Structure, chromosomal localization, tissue distribution, and receptor specificity

(nerve growth factor/brain-derived neurotrophic factor/neurotrophin 3/trk receptor/processing)

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ABSTRACT **Nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 (NT-3) are the three members of the neurotrophin family known to exist in mammals. Recently, a fourth neurotrophin (designated neurotrophin-4 or NT-4), which shares all of the features found in the mammalian neurotrophins, has been identified in *Xenopus* and viper. We used sequences specific to the *Xenopus*/viper NT-4 to isolate a neurotrophin from both human and rat genomic DNA that appears to represent the mammalian counterpart of *Xenopus*/viper NT-4. Human NT-4 as well as a human NT-4 pseudogene colocalize to chromosome 19 band q13.3. Mammalian NT-4 has many unusual features compared to the previously identified neurotrophins and is less conserved evolutionarily than the other neurotrophins. However, mammalian NT-4 displays bioactivity and trk receptor specificity similar to that of *Xenopus* NT-4.**

Nerve growth factor (NGF) is the prototypical member of a family of mammalian neuronal survival and differentiation factors (1), known as the neurotrophins, that also includes brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (2–10). These three neurotrophins are all initially synthesized as larger precursors that are proteolytically cleaved to release the mature neurotrophins. The mature regions of the three neurotrophins display 50–55% amino acid identity to each other, with the major regions of structural similarity bordering six invariant cysteine residues that, in active NGF, have been shown to form three intrachain disulfide bonds.

The neurotrophins can be distinguished based on their distinct patterns of spatial and temporal expression (11–15) as well as their differing effects on neuronal targets (3–7). The ability of a cell to respond to a particular neurotrophin appears to be dependent on the presence of the appropriate trk receptor. The three known trk receptors (designated here as trkA, trkB, and trkC) are transmembrane tyrosine protein kinases that specifically bind to the neurotrophins; trkA binds and can be activated by NGF and NT-3 (16–20), trkB binds and can mediate functional responses to BDNF and NT-3 (21–24), and trkC seems relatively specific for NT-3 (25).

Recently, a fourth neurotrophin (designated neurotrophin-4 or NT-4) has been molecularly cloned from *Xenopus* (26); a gene segment encoding part of the mature NT-4 from viper was also isolated. *Xenopus* NT-4 shares all the important features that characterize the mammalian neurotrophins. Here we describe the molecular cloning and characterization of a neurotrophin that apparently corresponds to the mammalian counterpart of *Xenopus*/viper NT-4.§ (The D-number assignment for humNT-4 chromosomal location is D19S202E.)

MATERIALS AND METHODS

Isolation and Sequencing of Genomic and cDNA Clones. Human and rat genomic DNA libraries (EMBL3/SP6/T7 vector) and a human prostate cDNA library (λgt10 vector) were purchased from Clontech and screened with the indicated probes as described (4, 10). DNA sequencing was performed manually (10) and using an automated system (model 373A; Applied Biosystems).

DNA Amplifications, Northern and Southern Blotting. Amplification reactions, blotting, and hybridization procedures were performed as described (4, 10, 11).

COS Cell Transfections, Immunoblotting, Neuronal and NIH 3T3 Assays, and Tyrosine Phosphorylation Analysis. The pCMX vector and transfections into COS cells have been described (27), except that the COS cells were maintained in defined medium conditions as described for the NIH 3T3 assay (23). Sensory neuron survival assays (28), trk-expressing NIH 3T3 assays (23), immunoprecipitations, and immunoblotting (23) were performed as described; the polyclonal antiserum recognizing NT-4 was generated against recombinant NT-3 and was provided by Amgen.

Fluorescence *in Situ* Chromosomal Hybridizations. Two genomic phage clones with inserts >10 kilobases (kb), containing the human NT-4 gene or the human NT-4 pseudogene, were used to prepare biotin-labeled probes for chromosomal hybridizations as described (29).

RESULTS

Cloning of Mammalian NT-4. The *Xenopus* NT-4 gene, even under reduced stringency conditions, was not able to identify specifically hybridizing sequences in mammalian genomic DNA; under these conditions, the human BDNF, NT-3, and NGF genes all identified their counterparts in *Xenopus* genomic DNA. This suggested that the mammalian NT-4 gene (if it existed) was much less conserved to its *Xenopus* counterpart than the other neurotrophins were to theirs. To clone a potentially more divergent mammalian homolog of NT-4, we performed PCRs on human and rat genomic DNA by using an upstream degenerate oligonucleotide primer corresponding to a region conserved in all the known neurotrophins [QYF(F/Y)ET], combined with a

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Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; NT-3 and NT-4, neurotrophins 3 and 4.

§The sequences reported in this paper have been deposited in the GenBank data base [accession nos. M86528 (humNT-4), M86529 (psiNI-4), and M86742 (ratNT-4)].

downstream degenerate oligonucleotide primer specific for a *Xenopus*/viper NT-4 sequence (CKAKQS) and potentially conserved between distantly related NT-4 genes because it borders one of the six absolutely conserved cysteine residues. Sequencing of the subcloned fragments derived from these amplification reactions revealed a neurotrophin apparently representing a mammalian homolog to *Xenopus*/viper NT-4; primary and secondary amplifications using other degenerate primers, also specific for *Xenopus*/viper NT-4 sequences, did not result in identification of additional mammalian homologs. Probes generated from amplified fragments corresponding to the putative mammalian NT-4 homolog were used to screen genomic DNA libraries, resulting in isolation of the genes apparently encoding human and rat NT-4, as well as a human NT-4 pseudogene.

Structure of Mammalian NT-4. Analysis of the putative mammalian NT-4 gene sequences predicts important structural similarities with the previously described neurotrophin precursors but also unusual differences. The presumptive initiation codon for the human and rat NT-4 precursors is surrounded by features (such as a splice acceptor site, an invariant upstream valine codon, and an invariant downstream leucine codon; Fig. 1) that characterize the known neurotrophins and are consistent with the existence of a second, longer, precursor form (4, 10). As is the case with the previously described neurotrophins, the human and rat NT-4 initiation codon is followed by a presumptive signal peptide sequence, a presumptive pro region, which contains all the invariant residues described for the other neurotrophins (including an N-linked glycosylation site), and an apparent dibasic cleavage site, which is followed by the sequence of the mature NT-4 (Fig. 1). However, the presumptive pro region for both human and rat NT-4 is notably shorter (by ≈60 amino acids) than the other known pro regions. The missing 60 amino acids correspond to the poorly conserved N-terminal region of the neurotrophin pro regions; removal of this region from the NGF precursor still allows correct processing in COS cells (30), as does the truncated NT-4 pro region (see below).

The mature human and rat NT-4 sequences are 95% identical at the amino acid level and share important features (such as the absolutely conserved cysteines) with the previously described neurotrophins, although they are unusual in containing a seven-amino acid insertion located between the second and third cysteines. The human and rat NT-4 sequences are substantially more related to *Xenopus* NT-4 (65% amino acid identity) than they are to BDNF (54%), NT-3 (52%), and NGF (50%) (Fig. 1*B*). After the seven-amino acid insertion, the mammalian NT-4 sequences are even more specifically related to their *Xenopus* homolog (84% identity) than to the other neurotrophins (65%, 62%, and 51% identity to BDNF, NT-3, and NGF, respectively), suggesting that selective forces are operating more strongly on the C terminus of this neurotrophin. Altogether, evidence suggests that

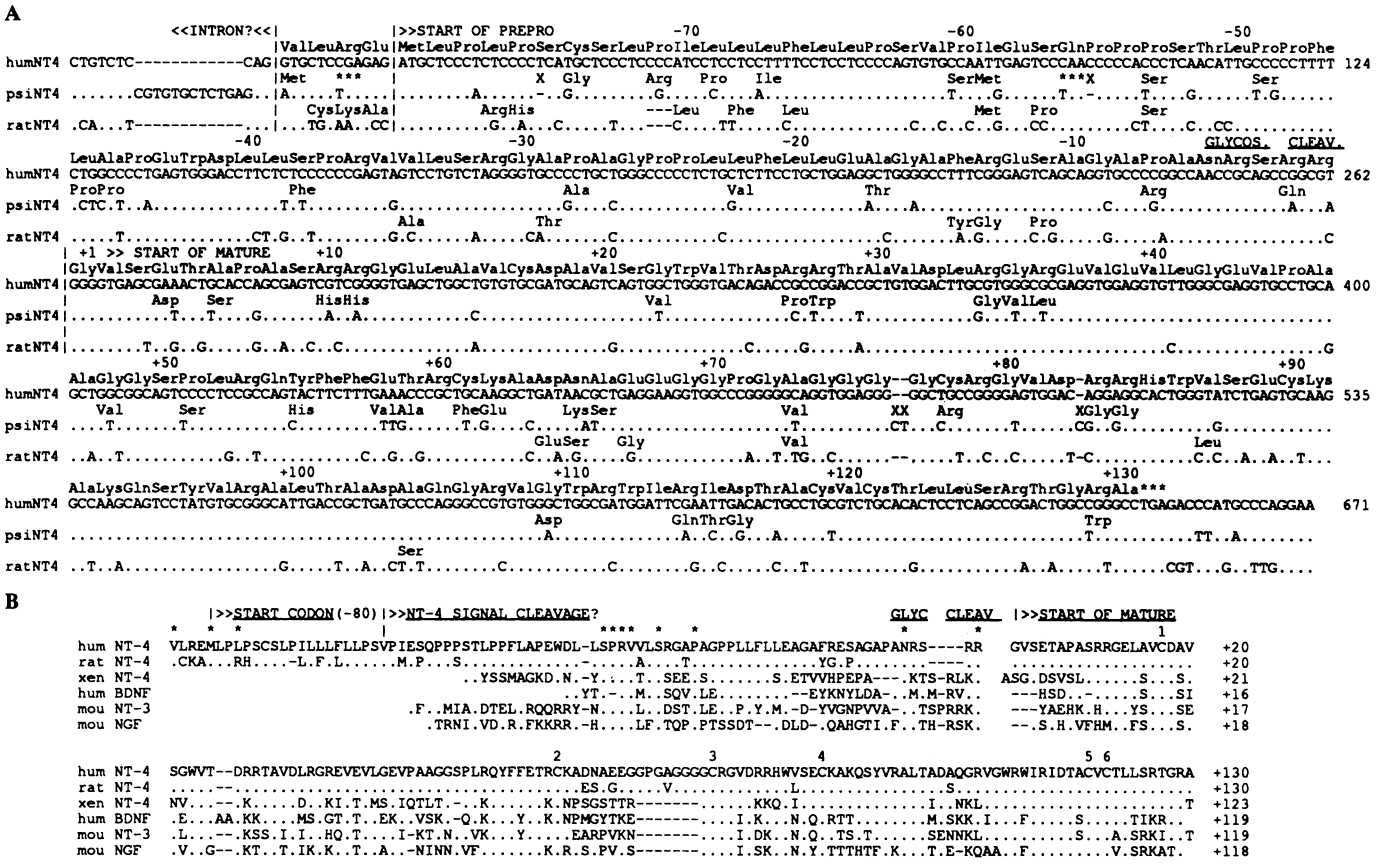

FIG. 1. (*A*) Comparison of human and rat NT-4 nucleotide and amino acid sequences and alignments with human NT-4 pseudogene (psiNT4). Sequences obtained from human and rat genomic DNA clones are shown. Identities to the human NT-4 DNA sequence are indicated by a dot, gaps are indicated by a dash, and only amino acid differences with the human NT-4 sequence are indicated for psiNT4 and rat NT-4; frameshifts (X) and stop codons (***) are marked. The sequence of a human NT-4 partial cDNA obtained from a prostate cDNA library exactly matched the human NT-4 genomic sequence from nucleotide 62 to the end of the indicated sequence. (*B*) Alignment of human and rat NT-4 protein sequence with *Xenopus* NT-4, human BDNF, mouse NT-3, and mouse NGF. The short pro region of human NT-4 could be aligned only with the C-terminal portions of the pro regions of the other neurotrophins. Residues in the prepro region, which are invariant among all neurotrophins, are marked with an asterisk above the residue. Identities with the human NT-4 sequence are indicated by a dot, and gaps are indicated by a dash. The cysteines in the mature region are numbered 1–6. Percentage identities provided in text ignore the seven-amino acid insertion; alignments were performed by using MacVector 3.5 (International Biotechnologies) and were refined visually.

we have isolated the true mammalian counterpart of *Xenopus* NT-4 (see *Discussion*); the mammalian NT-4 sequences are even more related to viper NT-4 (C.F.I. and H.P., unpublished results) than they are to the *Xenopus* NT-4 sequence.

Genomic library screening also resulted in the isolation of a second human sequence with significant homology to human and rat NT-4. However, this sequence is incapable of encoding a functional neurotrophin—it contains multiple frameshifts and an internal stop codon, and it lacks conserved features characteristic of neurotrophins such as the cleavage site which is required to release the mature form and two of the absolutely conserved cysteines (Fig. 1*A*). Thus, this sequence apparently corresponds to a human NT-4 pseudogene (psiNT4).

Chromosomal Localization of Human NT-4 and Its Pseudogene. Probes derived from the human and rat NT-4 genes identified the same unique *Eco*RI restriction fragments in human, rat, and mouse genomic DNA (data not shown), suggesting that there is a single mammalian gene for NT-4 and that close mammalian relatives do not exist. The finding that probes for both the human NT-4 gene and its pseudogene identified the same restriction fragment in human genomic DNA (data not shown) raised the possibility that NT-4 and its pseudogene are closely linked in the genome. To address this issue, and to determine the chromosomal localization of human NT-4, we performed fluorescence *in situ* hybridization on normal human metaphase chromosomes with biotin-labeled probes corresponding to both human NT-4 and the human NT-4 pseudogene. The human NT-4 probe alone (Fig. 2 *a–c*), or in combination with the pseudogene probe (Fig. 2*d*), resulted in a single fluorescent signal at chromosome 19, band q13.3, colocalizing both genes and suggesting that the NT-4 pseudogene arose via a recent gene duplication event. The other neurotrophins, despite sharing structural and functional homologies, are dispersed to different human chromosomes (10).

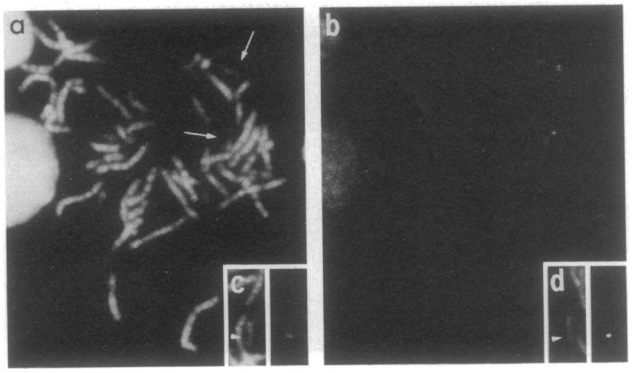

FIG. 2. Chromosomal localization of human NT-4 gene and its pseudogene. (*a*) Metaphase cell counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride. (*b*) Fluorescence photomicrograph of the metaphase cell in *a*, depicting hybridization of the human NT-4 probe to chromosome 19, band q13.3. The chromosome 19 homologs are identified with arrows. (*c*) Partial karyotype of a chromosome 19 homolog probed with human NT-4, illustrating specific labeling at 19q13.3 (arrowhead). Specific labeling of 19q13.3 was observed on one (1 cell), two (1 cell), three (10 cells), or all four (13 cells) chromatids of the chromosome 19 homologs in 25 cells examined. (*d*) Partial karyotype of a chromosome 19 homolog that was cohybridized with the NT-4 gene and NT-4 pseudogene probes, illustrating a single hybridization signal at 19q13.3 (arrowhead). Specific labeling of 19q13.3 was observed on two (6 cells), three (9 cells), or all four (10 cells) chromatids of the chromosome 19 homologs in 25 cells examined. Hybridization of the NT-4 pseudogene probe alone also resulted in specific labeling of 19q13.3 (data not shown). For both *c* and *d*, similar results were obtained in two additional hybridization experiments.

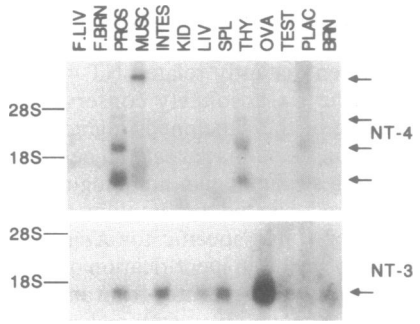

FIG. 3. Northern blot of human RNAs (enriched for polyadenylylated species) hybridized with human NT-4 and human NT-3 probes. Arrows on the right indicate predominant transcripts; rRNAs are indicated on the left. F.LIV, fetal liver; F.BRN, fetal brain; PROS, prostate; MUSC, muscle; INTES, small intestine; KID, kidney; LIV, liver; SPL, spleen; THY, thymus; OVA, ovary; TEST, testis; PLAC, placenta; BRN, adult brain.

Tissue Distribution of Human NT-4 Transcripts. To begin to explore the sites of NT-4 synthesis, we hybridized a human NT-4 probe to RNA prepared from a variety of fetal and adult human tissues (Fig. 3). Four distinct NT-4 hybridizing transcripts (approximate sizes, 1.1, 2.1, 4.0, and 9 kb) were identified, but not in brain and only in a limited number of peripheral tissues. The highest levels were found in prostate; lower levels were found in thymus, placenta, and skeletal muscle; and barely detectable levels were found in testis. The three smaller transcripts were detected in all tissues that expressed detectable levels of NT-4 transcripts, while the largest NT-4-hybridizing transcript was found only in skeletal muscle and testis. In contrast to human NT-4, human NT-3 is widely distributed, with strikingly high levels in the ovary (Fig. 3). The tissue distributions of both human NT-3 and NT-4 transcripts appear to be quite different from those described for the *Xenopus* counterparts (26); in *Xenopus*, it is NT-4 that is expressed at the highest levels in the ovary, while NT-3 transcripts are not detectable in the ovary.

Nucleotide sequencing of a NT-4-hybridizing cDNA clone obtained from a human prostate cDNA library revealed that it was identical in sequence to the human NT-4 gene (Fig. 1*A*), verifying that the observed Northern transcripts actually encode human NT-4.

Functional Expression and Neurotrophic Activity of NT-4. To obtain active human NT-4 protein, we transfected a human NT-4 expression construct into COS cells. This transfection revealed, as expected, an NT-4 precursor (≈27 kDa) notably smaller than the NT-3 precursor, as well as mature NT-4 (≈14 kDa) just larger than mature NT-3 (Fig. 4*A*). NT-4, however, has a preponderance of precursor to mature protein when expressed in COS cells, suggesting an inefficient processing mechanism for NT-4 in COS cells. This is also the case for BDNF; replacing the BDNF prepro region with that of NGF results in efficient processing in COS cells (S.P.S., D.R.G., N.Y.I., and G.D.Y., unpublished data). Similarly, utilizing a NT-4 expression vector in which the prepro region of human NT-4 is replaced with the prepro region of either NT-3 or *Xenopus* NT-4 results in a precursor similar in size to that of NT-3, with increased production of mature NT-4 (Fig. 4*A*, lane 4).

Like *Xenopus* NT-4 (26), human NT-4 supported survival and neurite outgrowth of sensory neurons from embryonic chicken dorsal root ganglia (Fig. 4*B*) but did not have notable effects on embryonic day 8 sympathetic ganglia (data not shown).

Human and *Xenopus* NT-4 Both Act Strongly on the trkB Receptor and Weakly on trkA. To determine whether NT-4 can utilize any of the known members of the trk family and

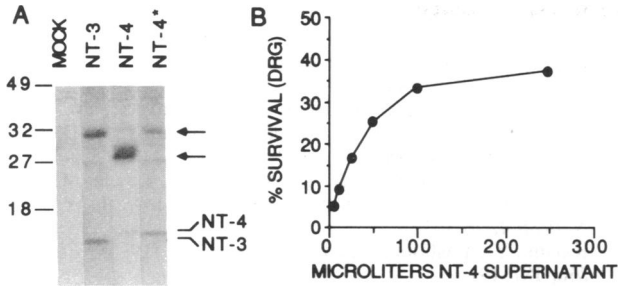

FIG. 4. Production of human NT-4 protein and activity on sensory neurons. (*A*) Immunoblot of supernatants from COS cells with an antibody that recognizes both NT-3 and NT-4, after mock transfection (MOCK) and after transfection with pCMX-NT-3 (NT-3), pCMX-NT-4 (NT-4), or an expression construct in which the prepro region of NT-4 was replaced with the prepro region of NT-3 (NT-4*). Size markers (kDa) are indicated on the left, with the positions of mature NT-3 and NT-4 proteins indicated on the right; upper arrow on the right indicates position of NT-3 and NT-4* precursors, while lower arrow marks position of NT-4 precursor. (*B*) Survival assay of highly enriched sensory neurons [isolated from dorsal root ganglia (DRG) of embryonic day 8 chickens] after exposure to increasing amounts of supernatant from COS cells transfected with human NT-4 in the pCMX expression vector. Total volume of culture medium was 2 ml; in the same assay, 250 μl of supernatants from COS cells transfected with MOCK, NGF, BDNF, and NT-3 expression constructs supported survival of 8.9%, 60%, 21%, and 36% of the sensory neurons, respectively.

to compare the trk specificity of human NT-4 with that of *Xenopus* NT-4, we used a recently described NIH 3T3 assay system in which introduction of trkB allows either BDNF or NT-3 to substitute for fibroblast growth factor as a survival/proliferation factor (23), while introduction of trkA allows NGF to act as a survival/proliferation factor (D. Glass, D.R.G., P. Hantzopoulos, M. Macchi, and G.D.Y., unpublished results); in contrast to another study (17), NT-3 has only a minor effect on these trkA-expressing NIH 3T3 cells.

The addition of NGF, BDNF, or NT-3 to either trkA- or trkB-expressing NIH 3T3 cells reveals striking effects—there is an obvious increase in the number of trkA-expressing cells when exposed to NGF (Fig. 5*A*), while BDNF or NT-3 results in a dramatic increase in the number of trkB-expressing NIH 3T3 cells (Fig. 5*B*). Supernatants from COS cells containing either human or *Xenopus* NT-4 had only a minor effect on cell numbers for trkA-expressing cells (similar to that of NT-3) but a striking effect on cell numbers for trkB-expressing cells (Fig. 5). Quantitative dose–response curves for survival and proliferation, as well as a precise comparison to the other neurotrophins, awaits purification of the NT-4 protein; the action of NT-4 on trkC remains to be determined.

Correlating with the dramatic effect of NT-4 on cell number in trkB-expressing NIH 3T3 cells, NT-4 very strongly induced the tyrosine phosphorylation of trkB; by comparison, equivalent amounts of NT-4 only weakly induced phosphorylation of the trkA receptor (Fig. 6).

DISCUSSION

We have described the molecular cloning and characterization of a neurotrophin apparently representing the mammalian counterpart of *Xenopus*/viper NT-4. Structurally, mammalian NT-4 is quite unusual compared to the previously described neurotrophins because of a truncated pro region and a seven-amino acid insertion within its mature region. Truncation of the human NT-4 pro region is not necessarily responsible for inefficient processing in COS cells because BDNF, despite a pro region of normal length, displays a similar processing deficit. Although it remains to be established whether NT-4 and BDNF are efficiently processed *in*

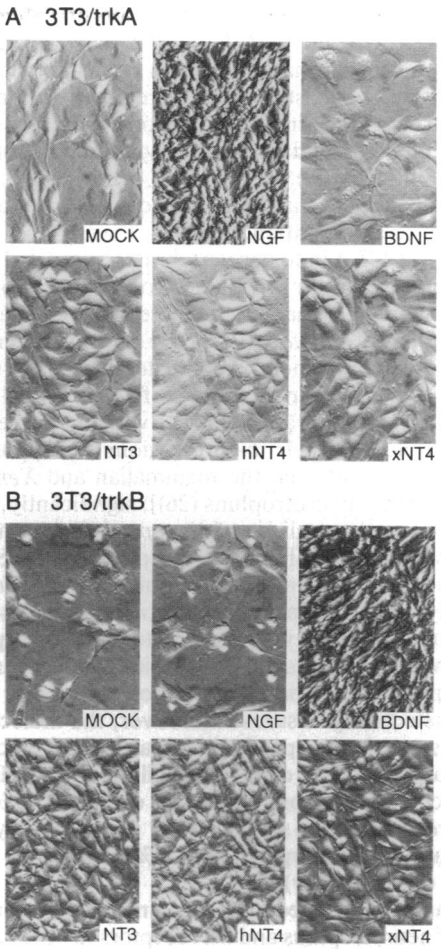

FIG. 5. Comparison of human and *Xenopus* NT-4 actions on NIH 3T3 cells expressing trkA or trkB. (*A*) NIH 3T3 cells expressing trkA were grown in serum-free, defined medium conditions supplemented with 5 nM NGF, BDNF, and NT-3, or with supernatants from COS cells (also grown in defined medium) transfected with MOCK (1:10 dilution), human (h) NT-4 (1:10 dilution), or *Xenopus* (x) NT-4 (1:50 dilution) expression constructs for 3–4 days. (*B*) NIH 3T3 cells expressing trkB were grown in serum-free, defined medium conditions supplemented with 5 nM NGF, BDNF, and NT-3, or with supernatants from COS cells (also grown in defined medium) transfected with MOCK (1:20 dilution), human NT-4 (1:20 dilution), or *Xenopus* NT-4 (1:50 dilution) expression constructs for 3–4 days.

vivo, production of mature BDNF or NT-4 in COS cells can be increased by replacing the naturally occurring BDNF or

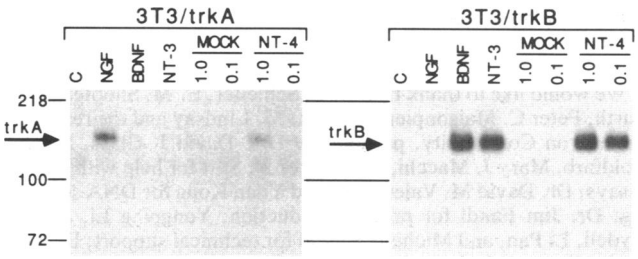

FIG. 6. Human NT-4 weakly induces tyrosine phosphorylation of trkA and strongly induces tyrosine phosphorylation of trkB. NIH 3T3 cells expressing either trkA or trkB were exposed to either 2 nM NGF, BDNF, and NT-3, or to supernatants from COS cells transfected with mock or human NT-4 expression constructs; the COS supernatants were used both undiluted (1.0) and at a 1:10 dilution (0.1). After a 5-min exposure, the cells were rapidly lysed, immunoprecipitated with a polyclonal antibody that recognizes both trkA and trkB, and then immunoblotted with an anti-phosphotyrosine antibody.

NT-4 pro regions with those of other neurotrophins (such as NGF, NT-3, or *Xenopus* NT-4). The seven-amino acid insertion found in the mature portion of mammalian NT-4 is located within the least conserved region even among individual neurotrophins, in a surface loop apparently capable of tolerating changes and insertions without affecting overall neurotrophin structure (31); lack of evolutionary conservation would suggest that this surface loop is not directly involved in receptor interactions.

Analysis of NT-4 in lower vertebrates suggested that NT-4 was diverging at a faster rate than the other neurotrophins (26), consistent with our inability to detect a mammalian NT-4 homolog using a *Xenopus* NT-4 probe at low stringency. Thus, it seems likely that the neurotrophin described here represents the closest mammalian homolog of *Xenopus* NT-4, even though the mammalian and *Xenopus* versions share only 65% identity throughout their mature regions [as opposed to the 85–90% identity between the mammalian and *Xenopus* versions of the other neurotrophins (26)]; significantly, the C-terminal halves of mammalian and *Xenopus* NT-4 are much more highly conserved (84% identity). Despite differences in tissue distributions, the similar neuronal and trk receptor specificities displayed by human and *Xenopus* NT-4 suggest that they may be functional, as well as structural, counterparts. The NT-4 example highlights the fact that individual neurotrophins (or even portions of the same neurotrophin) are subject to different selective pressures. Moreover, the molecular clock also seems to vary its speed within particular branches of the evolutionary tree. For example, while NGF and BDNF are similarly conserved from human to chicken (90% and 93% identity, respectively), salmon NGF is remarkably diverged from its human counterpart (only 56% identity) as compared to BDNF (95% identity) (26).

Important issues raised by our findings concern the ultimate number of neurotrophins and trk receptors to be found, as well as the physiological relevance of having a system in which multiple factors have overlapping specificities for the same set of receptors. The similar trk receptor specificities of human and *Xenopus* NT-4 suggest that selection is simultaneously operating on both the factors and their receptors, maintaining important functional interactions. However, the differing distributions of a given neurotrophin in lower vertebrates as compared to mammals suggest that these molecules may acquire new roles during the course of evolution. The multiplicity and physiological significance of these roles and receptor interactions presumably determine the extent to which the various neurotrophins are evolutionarily conserved.

Note Added in Proof. While this manuscript was under review, Berkemeier *et al.* (32) reported the cloning of the same human and rat neurotrophin gene, which was designated NT-5. Based on the arguments presented in our paper, we believe the nomenclature should be NT-4.

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