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Rat Ciliary Neurotrophic Factor (CNTF): Gene Structure and Regulation of mRNA Levels in Glial Cell Cultures

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KEY WORDS Astrocytes, Schwann cells, Interferon-γ, Fibroblast growth factor, Cyclic AMP

ABSTRACT The structure of the rat ciliary neurotrophic factor (CNTF) gene and the regulation of CNTF mRNA levels in cultured glial cells were investigated. The rat mRNA is encoded by a simple two-exon transcription unit. Sequence analysis of the region upstream of the transcription start-site did not reveal a typical TATA-box consensus sequence. Low levels of CNTF mRNA were detected in cultured Schwann cells, and CNTF mRNA was not increased by a variety of treatments. Three-week-old astrocyte-enriched cell cultures from new-born rat brain contained easily detectable CNTF mRNA. In astrocyte-enriched cultures, upregulation of CNTF mRNA levels was observed after treatment with IFN-γ. CNTF mRNA levels were down-regulated in these cells by treatments that elevate intracellular cyclic AMP and by members of the fibroblast growth factor (FGF) family. The implications of these results for potential in vivo functions of CNTF are discussed.

INTRODUCTION

Ciliary neurotrophic factor (CNTF) is a 22 kDa acidic cytosolic protein that was originally identified and partially purified as target-derived neurotrophic factor for chick ciliary parasympathetic neurons; hence its name (Barbin et al., 1984; Manthorpe et al., 1986). In the course of further investigations performed with CNTF purified to homogeneity and also recombinant CNTF, it became apparent that the spectrum of biological actions of CNTF is much broader. CNTF proved to be a powerful neurotrophic molecule not only for chick E8 parasympathetic cholinergic neurons but also for E11 sympathetic and E10 sensory neurons (Barbin et al., 1984), chick E6 spinal motoneurons (Arakawa et al., 1990), chick E8 nodose ganglion neurons, and E10 trigeminal neurons (Sendtner et al., 1991). Moreover, it was shown that CNTF induces cholinergic properties in rat sympathetic neurons in culture as reflected by an increase in choline acetyltransferase and a reduction of tyrosine hydroxylase (Saadat et al., 1989) as well as inducing differentiation and VIP expression by chick sympathetic neuronal precursors (Ernsberger et al., 1989). CNTF has also been shown to induce differentiation of rat optic nerve O-2A progenitor cells to type-2 astrocytes (Lillien et al., 1988), induce acute-phase protein expression in hepatocytes (Schooltink et al., 1993), activate transcription of neuropeptide genes in neuroblastoma cells (Symes et al., 1993), and promote survival and maturation of cultured oligodendrocytes (Bergsteindottir et al., 1993).

The very broad spectrum of biological actions predominantly demonstrated in vitro but also in vivo (Op-

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Abbreviations: Act D, actinomycin D; 8-br-cAMP, 8-bromo-adenosine-cAMP; dibu-cAMP, dibutyryl-adenosine-cAMP; CGRP, calcitonin gene related peptide; CNTF, ciliary neurotrophic factor; CHX, cycloheximide; Dex, dexamethasone; EGF, epidermal growth factor; FGF, fibroblast growth factor; FK, forskolin; IFN, interferon; IFG-I, insulin-like growth factor I; IL-1β, Interkeukin-1β; IL-6, interleukin-6; KA, kainic acid; PDGF-AB, platelet derived growth factor AB; RA, all trans retinoic acid; SC CM, Schwann cell conditioned medium; SC co-cult, Schwann cell co-culture; Sub P, substance P; T3, 3,3',5-triiodo-L-thyronine; TGF-β_1, platelet transforming growth factor β1; TPA, phorbol 12-myristate 13-acetate; VIP, vasoactive intestinal peptide.

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penheim et al., 1991; Sendtner et al., 1990, 1992b), after local or systemic administration, is in distinct contrast to the very restricted expression of CNTF exclusively to the postnatal period and confined to myelinating Schwann cells and a subpopulation of type-1 astrocytes (Stöckli et al., 1991). Besides evaluation of the physiological function of CNTF by gene targeting and its potential patho-physiological function as a lesion factor, the elucidation of the regulation of its synthesis and the restricted cellular expression represent a most important step on the way to a comprehensive understanding of the physiological roles of CNTF. With this in mind, we analysed the genomic organisation of the CNTF gene, characterised its promoter region and investigated the regulation of CNTF synthesis in cultured Schwann cells and astrocytes under a great variety of experimental conditions.

MATERIALS AND METHODS

Materials

Actinomycin D, forskolin (7β-acetoxy-8,13-epoxy-1 α,6β,9α-trihydroxylabd-14-ene-11-one), 1,9-dideoxy-forskolin, cytosine-β-D-arabinofuranoside (ara-C), norepinephrine, isoproterenol, 8-bromo-adenosine-cAMP (8-br-cAMP), dibutyryl-adenosine-cAMP (db-cAMP), mouse epidermal growth factor (EGF), ionomycin, 3,3′-5-triiodo-L-thyronine (T3), all trans retinoic acid (RA), calcitonin gene related peptide (CGRP), glucagon, methionine enkephalin (Met-enk), phorbol 12-myristate 13-acetate (TPA), cycloheximide (CHX), kainic acid (KA), dexamethasone (Dex), histamine, and testosterone were purchased from Sigma Chemical Co. (St. Louis). Rat recombinant basic FGF (bFGF), human recombinant IL-6, human plasma thrombin, okadaic acid, and substance P were purchased from Boehringer Mannheim. Porcine platelet transforming growth factor βI (TGF-β_1) was purchased from R&D Systems, Inc. Human platelet derived growth factor AB (PDGF-AB) was from British Biotechnology (Oxford). Rat recombinant interferons α and β were purchased from Bioferon Gmbh. Interleukin-1β (IL-1β) was a gift from Dr. A. Gronenborn (Martinsried) by courtesy of Biogen (Geneva). Human recombinant insulin-like growth factor I (IGF-I) was generously provided by Dr. A. Skottner (Kabi Peptide Hormone, Stockholm). Fibroblast growth factor 5 (FGF-5) and kFGF (also called FGF-4) were gifts from Mitchell Goldfarb and Claudio Basilio, respectively (Columbia University, NY). Cell culture media and trypsin-EDTA were from Gibco/BRL.

Isolation of Genomic Clones

Genomic Southern blots of rat DNA were hybridised against a variety of degenerate oligonucleotides whose sequence was based on peptide sequences of purified rat CNTF (M. Sendtner and F. Lottspeich, Martinsried). One oligonucleotide 33-mer, LP3 (5′-CTCCTCIAGCTGRTAIGCAAAKGRCTIACCTG-3′, based on the CNTF peptide sequence QVSAFAYQLEEL) detected a single band of 5 kb in an EcoRI digest which was also visible amongst the multiple bands detected by several other oligonucleotides based on different protein sequences. LP3 was used to screen a rat genomic bank of partial EcoRI-digested DNA in λ vector Charon 4A (Clontech). Hybridising clones were isolated and confirmed as CNTF by sequencing and by hybridisation against CNTF cDNA, which has been isolated in parallel by PCR methodology (Stöckli et al., 1989). Sequencing was performed by the Sanger dideoxy method on plasmid template.

Brain Astroglial Cell Culture

New-born Wistar rats were sacrificed and brains were dissected out. After removal of the meninges the tissue was cut into small pieces and treated with trypsin. Cells were mechanically dissociated and plated out in Costar 162 cm^2 culture flasks in 10% fetal calf serum (FCS) in DMEM. When the cells had almost reached confluence cytosine arabinoside (Ara-C, 20 μM) was added to the medium for 3 days to suppress fibroblast proliferation. Cells were grown for 2–3 weeks in Costar 162 cm^2 flasks, detached by trypsinisation and 3×10^5 cells were plated out on 6 cm dishes. After a further 3 to 7 days in culture the cells were treated for the indicated times (see Results) and used for RNA analysis.

Sciatic Nerve Schwann Cell Culture

Schwann cell cultures were prepared as described by Matsuoka et al. (1991). Briefly, sciatic nerves from newborn rats were treated with collagenase and then trypsin. After trituration the cells were plated out onto 6 cm dishes in DMEM/10% FCS. After two cycles of Ara-C treatment the cells were replated on poly-lysine coated dishes in DMEM/10% FCS and 10 μM forskolin to promote Schwann cell proliferation. Expanded Schwann cell cultures were kept for 1–3 weeks without forskolin before being used for experiments.

Primer Extension Assay

Poly A^+ RNA was prepared from 4-week-old cultures of rat brain astrocytes using the Quick-Prep mRNA purification kit (Pharmacia) following the manufacturers' instructions. The priming oligonucleotide was a 30-base antisense oligonucleotide (PEXT-1) from the 5′-end of the CNTF protein coding region: 5′-GAAGGGTCAGAGGTGTTTGCTCTGCGAAAG-3′ (bases 82–111 in the cDNA, Stöckli et al., 1989). PEXT-1 was 5′ end-labelled with ^{32}P-ATP and polynucleotide kinase. Hybridisation of primer and RNA was as described in Ausubel et al. (1990). The reverse transcription reaction was carried out in the following conditions: 10 μg poly A^+ RNA, 50 mM Tris (pH 8.3), 40 mM KCl, 7 mM

$MgCl_2$, 1 mM DTT, 50 μg/ml BSA, 1 mM dNTPs, and 20 U AMV reverse transcriptase (Life Sciences). After 30 min at 42°C, fresh enzyme was added and the reaction was continued at 50°C for 30 min. As a positive control a synthetic sense RNA transcript that extended from the Apa I site at −120 relative to the 5′-end of the longest cDNA clone (Stöckli et al., 1989) through the first exon, thus containing the binding site for the primer extension oligonucleotide, was made by in vitro transcription from the plasmid p64, a 4.5 kb ApaI/SalI fragment of the rat CNTF gene in the Bluescript vector. The primer extension product generated from this template would be ~200 bases. The samples were separated on a 6% sequencing gel alongside the products of a sequencing reaction generated from PEXT-1 with a rat CNTF genomic clone as template.

5′ RACE (Rapid Amplification of cDNA Ends)

The protocol described by Frohmann (1990) was followed. Reverse transcription was initiated from various antisense primers whose sequences were based on the 5′ sequences of rat CNTF cDNA using polyA$^+$ RNA from rat astrocyte cultures as template. The sequences of the oligonucleotides were as follows: Pal-2 (20-mer) 5′-TCAGTGCTTGCCACTGGTAC-3′; PEXT-1 (30-mer) 5′-GAAGGGTCAGAGGTGTTTGCTCTGCGAAAG-3′, PEXT-2 (30-mer) 5′-GGAAATAAGTGAGCTGGTTTGCTTCTGTGT-3′. The resulting cDNA was tailed with guanosine residues and after removal of priming oligos by Centricon-30 filtration, PCR was carried out using oligo-dC primer and primers upstream from the original reverse transcriptase priming oligonucleotides-"nested PCR." PCR products were either cloned into pUC19 vector and sequenced using the Sanger dideoxy method or sequenced directly using the Applied Biosystems automated sequencing machine.

RNA Isolation and Northern Blot Analysis

Cells (about 1 million) were treated as described in the Results section and RNA was isolated by the method of Chomczynski and Sacchi (1987). Briefly, after removing the medium and adding 0.5 ml solution D (4 M guanadinium isothiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% Sarkosyl and freshly added 0.1 M 2-mercaptoethanol) and a known amount (usually 10 pg) of recovery standard (a 600 bp synthetic sense CNTF RNA transcript), the mixture was collected by scraping into a microfuge tube containing 500 μl phenol, 100 μl chloroform/isoamyl alcohol (50/1), and 50 μl 2M sodium acetate (pH 4.1). The sample was vortexed, left on ice 10 min, centrifuged for 10 min, and the supernatant was re-extracted with 0.5 ml chloroform/isoamyl alcohol. RNA was precipitated by the addition of 0.5 ml isopropanol at −20°C for 1–2 h. After centrifugation the pellet was resuspended in solution D and again precipitated with isopropanol. Generally, about 20 μg total RNA was recovered from a confluent 6 cm plate.

RNA samples were separated on 1.5% agarose gels containing formaldehyde (Sambrook et al., 1989). Gels were vacuum-blotted onto Hybond-N membrane (Amersham).

Pre-hybridisation and hybridisation were carried out in 10% formamide; 5× SSC; 20 mM sodium phosphate, pH 7.0; 5× Denhardt's solution; 50 mM EDTA; 1% SDS, and 500 μg/ml denatured herring sperm DNA. The hybridisation temperature was 65°C. A ^{32}P-labelled probe (10^9 dpm/μg) was prepared by in vitro transcription of a CNTF protein-coding region template in appropriately linearized Bluescript plasmid. Blots were washed twice in 2× SSC/0.1% SDS at RT, once in the same buffer at 60°C for 30 min and once at 70°C in 0.1× SSC/0.5% SDS for 1 h.

The autoradiograph of the Northern blot showing increased CNTF mRNA expression in astrocyte-enriched cultures after IFN-γ treatment was scanned using an LKB laser densitometer.

RESULTS

Structure of the Rat CNTF Gene

The sequence of the rat CNTF gene and flanking regions is shown in Figure 1A,B. By comparison with the cDNA sequence (Stöckli et al., 1989), the CNTF gene is shown to be composed of two exons separated by an intron of 1 kb. A transcriptional start-site was identified by primer extension of a CNTF-specific oligonucleotide against cultured rat astrocyte poly A$^+$ RNA (Fig. 2A, lane 1). Primer extension using positive control sense RNA transcript generated the expected 200 base product (Fig. 2A, lane 2). The transcriptional start-site shown here corresponds with that suggested by cDNA cloning (Stöckli et al., 1989).

The existence of a longer ~4 kb RNA species that hybridised to a labelled CNTF RNA probe, in addition to the more abundant 1.2 kb mRNA (Rudge et al., 1992 and our unpublished observation), as well as the observation that in rabbit sciatic nerve CNTF mRNA is ~4 kb (Lin et al., 1989), led us to investigate in detail whether there are extensions of the rat CNTF mRNA at the 5′-end. 5′ RACE experiments were carried out using three antisense oligonucleotide specific for the 5′-end of the rat astrocyte CNTF mRNA (Fig. 2B). For all combinations of oligonucleotides, the size of the major PCR product corresponded to the transcriptional start predicted by both cDNA cloning and primer extension. All attempts to amplify higher molecular weight species that appeared in low amounts in some PCR reactions were unsuccessful. The PCR products of the 5′ RACE protocol were cloned and sequenced, the results of which further corroborated the previous findings (data not shown). Thus the 5′-untranslated region of rat CNTF mRNA is 75 bases long.

The promoter region does not contain a typical TATA box sequence. The sequence TAGAAAA at position −31, may serve this function. A transcriptional start-site for

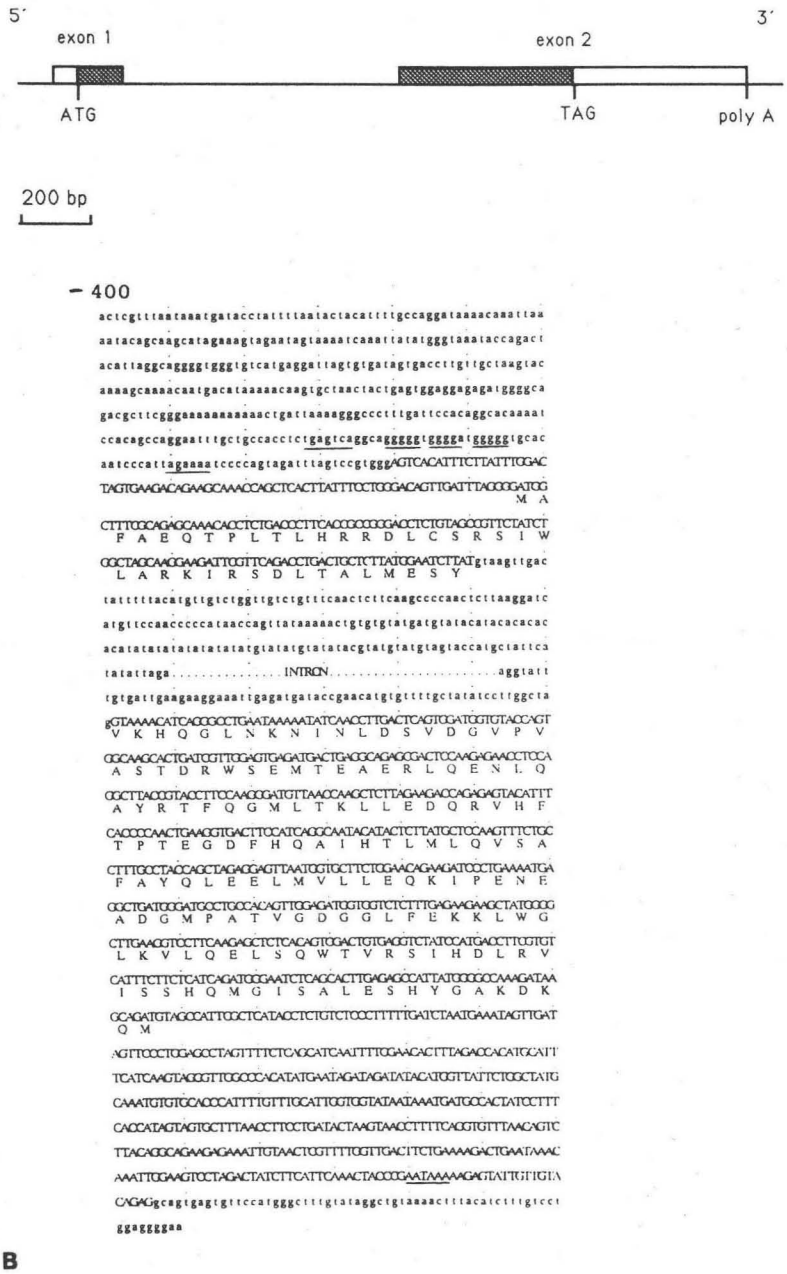
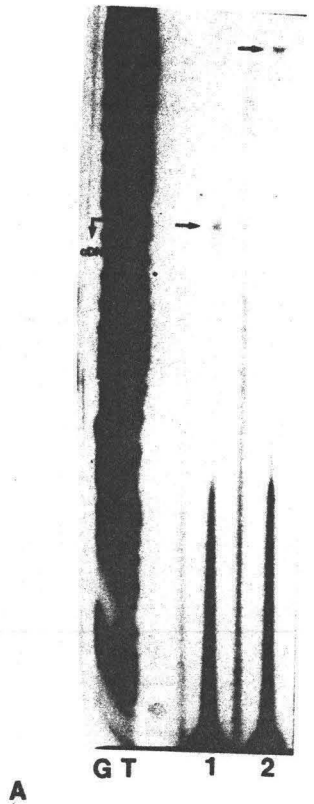
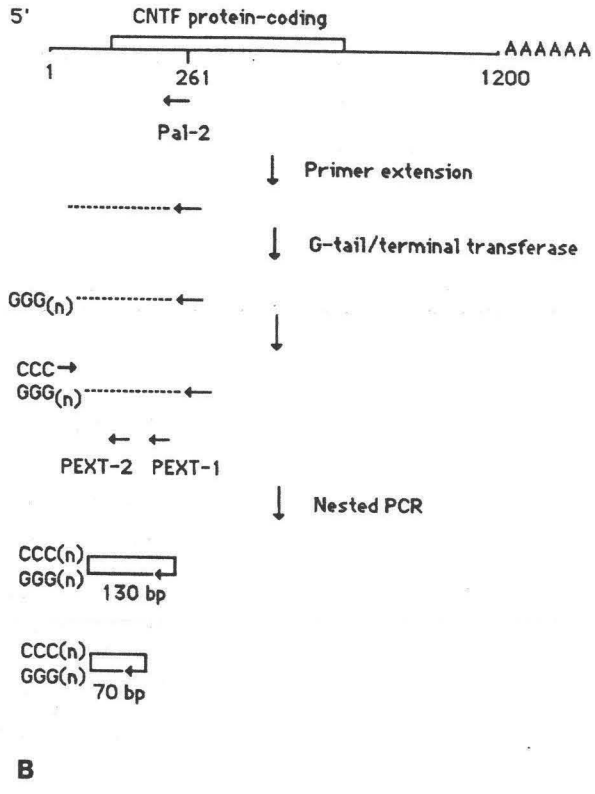


Fig. 1. **A:** Structure of the rat CNTF gene. Boxes represent the 2 exons and shaded areas show the CNTF protein-coding region. **B:** The sequence of the rat CNTF gene and derived CNTF protein sequence, partial intron, and 5′ and 3′ flanking sequences. Underlined sequences represent a potential AP-1 binding site, G-rich sequences, potential TATA-box sequence in the 5′-flanking region, and the polyadenylation signal AATAAA at the end of the gene.

the human CNTF gene has been suggested (Lam et al., 1991) which differs from that shown here for the rat gene. The human and rat gene sequences are compared in Figure 2C. The human mRNA would have a 5′ untranslated region of only four bases. A consensus AP-1 sequence, which mediates the transcriptional regulation functions of the *fos/jun* family of DNA binding proteins (for review, see Herschman, 1991), is found at −72 and is conserved in the human gene (Lam et al., 1991). A well-conserved G-rich sequence, found between −61 and −45, has the characteristics of a GT-box, which is a potential target sequence for the DNA binding protein SP-1 and the related GT box-binding proteins (Hagen et al., 1992; Kingsley and Winoto, 1992).





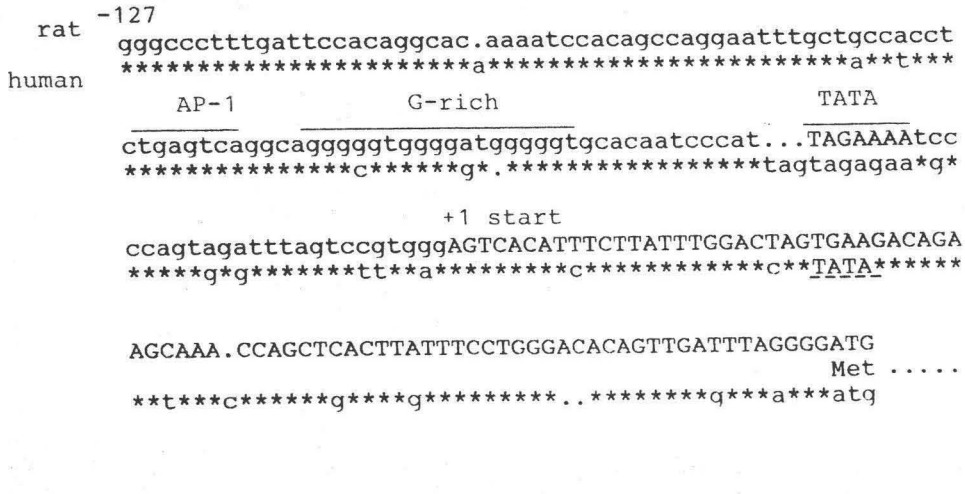


Fig. 2. A: Primer extension analysis of cultured rat brain poly A^+ RNA using a ^{32}P-labelled base CNTF antisense synthetic oligonucleotide (PEXT-1) encompassing bases 82 to 111 of the CNTF cDNA. G and T: sequencing reaction from PEXT-1 against a plasmid clone containing the rat CNTF promoter and first exon. Lane 1: primer extension using rat astrocyte poly A^+ as template. Lane 2: primer extension using an in vitro-synthesized positive control template RNA extending from position −120 relative to the proposed transcriptional start-site. Arrows indicate the primer extension products. Negative controls were (not shown) tRNA, adult rat liver total RNA, and a 30-base ^{32}P-labelled sense CNTF oligonucleotide as priming oligonucleotide. In all cases no extension products were observed. B: Diagram showing the position of oligonucleotides used for primer extension analysis and 5′ RACE protocol. Base numbering is derived from the rat cDNA sequence (Stöckli et al., 1989): Pal-2 (bases 243–261); PEXT-1 (bases 82-111); PEXT-2 (bases 28–57). C: Comparison of the sequences at the 5′-ends of the rat and human CNTF genes. Conserved AP-1, G-rich, and TATA box in the rat gene are indicated. The TATA box for the human gene proposed by Lam et al. (1991) is underlined by a broken line.

The sequences of the intron donor and acceptor sites correspond well to the consensus sequences suggested by Padgett et al. (1986).

Consensus donor: CAG↓GT(A/G)AGT
CNTF donor: TAT ↓ *GTAAGT*
Consensus acceptor: YYYYYYXCAG↓G
CNTF acceptor: *CCTT*GGCTA*G*↓ *G*

CNTF mRNA Expression in Cell Culture

A number of different cell types were tested by Northern blot analysis for CNTF mRNA expression including 3–4-week-old astrocyte-enriched cultures from newborn rat brain, primary Schwann cells, microglia-enriched cultures from new-born rat brain, C6 glioma, RN22 Schwannoma, NIH 3T3 fibroblasts, and primary fibroblasts from newborn rat sciatic nerve. Only primary astrocytes, Schwann cells and C6 glioma cells showed detectable levels of the 1.2 kb CNTF mRNA, astrocyte cultures expressing by far the highest levels of mRNA (Fig. 3A, data for all cell types not shown).

A wide range of treatments and factors were tested for their ability to alter CNTF mRNA levels in primary rat astrocytes (Table 1). Forskolin, cAMP analogues, isoproterenol, and norepinephrine reduced CNTF mRNA levels (Fig. 3A,D, data for norepinephrine not shown). The CNTF mRNA levels in astrocyte cultures were also down-regulated by four members of the fibroblast growth factor family. bFGF, aFGF (data not shown), k-FGF, and FGF-5 (Fig. 3B). Heparin was used to potentiate the activity of FGF-5 and k-FGF (Hughes et al., 1993; Delli-Bovi et al., 1988). Only treatment of astrocytes with γ-interferon resulted in a modest (3-fold, as measured by densitometry scanning) increase in CNTF mRNA levels (Fig. 3C).

The level of CNTF mRNA was very low in Schwann cells and no treatment was found which raised it above the basal levels. It may be possible that Schwann cells in culture down-regulate their own CNTF mRNA synthesis by production of diffusible or non-diffusible factor(s). To test whether such factor(s) could down-regulate CNTF mRNA levels in astrocytes, astrocytes were co-cultured with Schwann cells or in medium conditioned by Schwann cells. Neither of these treatments altered the levels of CNTF mRNA in astrocytes or Schwann cells (see Table 1).

Expression of CNTF mRNA in Astrocytes Derived From Different Brain Regions

Heterogeneity between cultured astrocytes derived from different brain regions in their GFAP content, glutamate uptake, responses to β-adrenergic stimulation (for review see Wilkin et al., 1990), and response to bFGF (Perraud et al., 1990) has been reported. In order to ascertain whether there are differences in CNTF mRNA expression in different astrocyte populations, we plated cells from cortex, hippocampus, and cerebellum and investigated CNTF mRNA expression and forskolin responsiveness by Northern blot analysis. CNTF mRNA was found in astrocytes of all of these brain regions (Fig. 4). Immunohistochemical analyses of these cultures using antibodies for GFAP and Thy 1 to determine the relative proportions of astrocyte and fibroblasts revealed differences from experiment to experiment, so that the differences in CNTF mRNA amounts between cortical, cerebellar, and hippocampal cultures shown in Figure 4 are not suggested to represent regional variation in CNTF expression. In all cases, CNTF mRNA levels were down-regulated by forskolin treatment (Fig. 4).

CNTF mRNA Half-Life Estimation

Astrocyte cultures were treated with either actinomycin D alone or forskolin and actinomycin D together. Densiometric scanning of the autoradiograph shown in Figure 5 showed that in the presence of the transcriptional inhibitor actinomycin D, CNTF mRNA levels had decreased to 50% by 6 h. Thus the half-life of the CNTF mRNA is 6 h. In the presence of forskolin, the decrease in CNTF mRNA levels was delayed, suggesting possible post-transcriptional effects of forskolin on CNTF mRNA.

DISCUSSION

The structure of the rat CNTF gene has been elucidated and the regulation of the CNTF mRNA was investigated in a number of cell types. The gene is split into two exons of 190 and 1,003 bp separated by an intron of about 1 kb. By primer extension and 5′-RACE methodology we show that rat CNTF mRNA has a 5′-untranslated region of 75 bases, of average length for eucaryotic genes (Kozak, 1987). If the ~4 kb RNA species that hybridises to a CNTF RNA probe in Northern blot analysis (Rudge et al., 1992) is generated from the CNTF gene it most probably is derived from alternative polyadenylation site usage.

The rat CNTF promoter region does not contain a typical TATA box, although the related sequence TAGAAAA is found at the appropriate position (~−30)relative to the transcriptional start-site, and this sequence may function as a site for transcription complex formation. A different transcriptional start-site has been proposed for the human CNTF (Lam et al., 1991), generating a 5′ untranslated region of only four bases. The shortest 5′-noncoding sequence as deduced from a survey of 699 vertebrate sequences is seven bases (Kozak, 1987). In three other cases where the noncoding sequence was less than ten bases multiple translation initiation sites were observed. The TATA box suggested for the human gene is not conserved in the rat. The rat, mouse, and human genes all contain the sequence TAGA at the −30 position relative to the transcription start-site suggested in this paper. Considering the above ob-

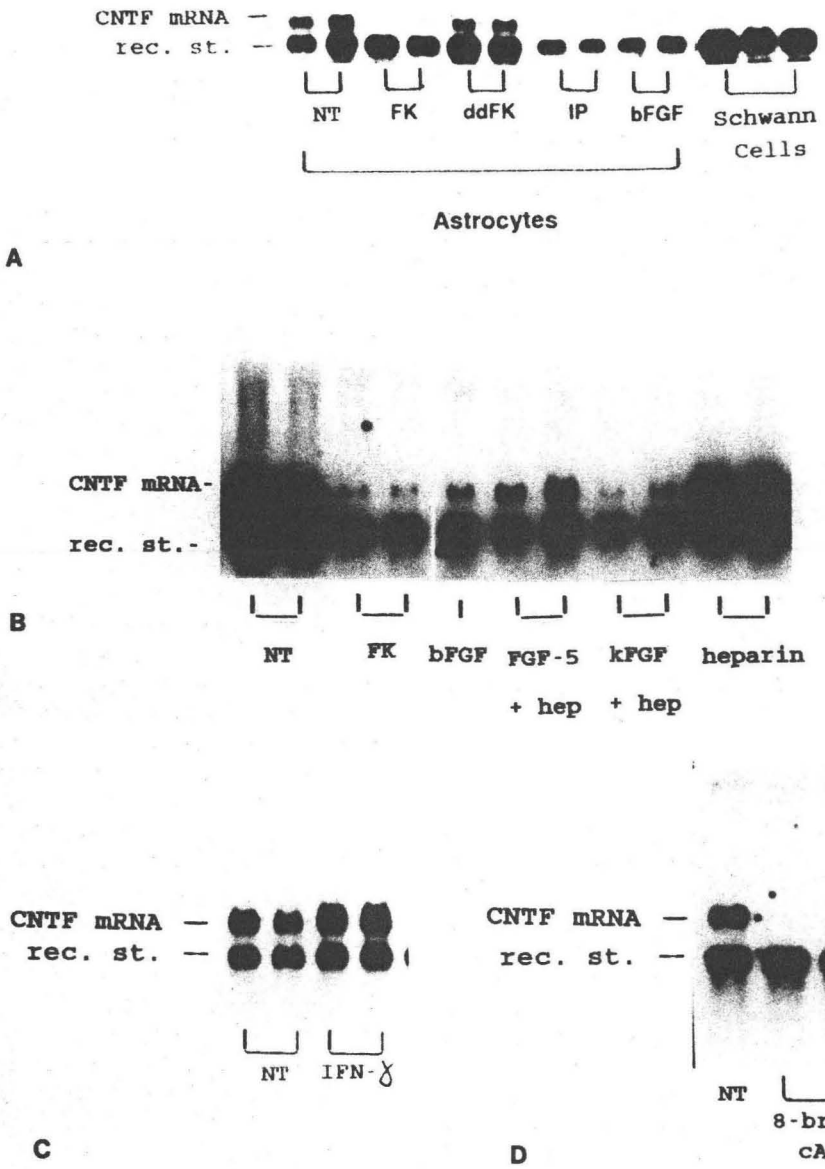


Fig. 3. A. Northern blot showing expression of CNTF mRNA in 3-week-old astrocyte-enriched cultures from newborn rat brain and in cultured rat Schwann cells. Recovery standard (rec. st.) was a 600 base sense CNTF in vitro transcript (10 pg/lane). NT, Untreated cells; FK, forskolin (10 μM); ddFK, dideoxy-forskolin (10 μM); IP, Isoproterenol (6.25 μM); bFGF, basic FGF (20 ng/ml medium). All treatments 24 h. B: Northern blot analysis of CNTF mRNA levels in 3-week-old astrocyte-enriched cultures from new-born rat brain. NT, untreated cells; FK, forskolin (10 μM); bFGF, human basic FGF (20 ng/ml); recombinant human FGF-5 (100 ng/ml) + heparin. Human kFGF (100 ng/ml) + heparin. Heparin (500 μg/ml). Treatment for 24 h. C: Northern blot analysis of CNTF mRNA levels in 3-week-old astrocyte-enriched cultures from new-born rat brain. NT, untreated cells; IFN-γ, interferon-γ (300 U/ml). Treatment for 24 h. D: Northern blot analysis of CNTF mRNA levels in 3-week-old astrocyte-enriched cultures from new-born rat brain. NT, untreated cells. 8-bromo cAMP (1 mM). Treatment for 24 h.

servations, the CNTF promoter may be included in the class of promoters that have atypical TATA boxes and are not highly GC-rich (Smale and Baltimore, 1989). In mutagenesis experiments, point mutations of the third position T of the TATA sequence to either G or A caused a reduction in transcriptional activity (Giangrande et al., 1989; Myers et al., 1986). Pugh and Tjian (1991) suggest that for promoters lacking a typical TATA box binding of the SP-1 protein at GC-rich sequences near the transcriptional start site may function to tether the transcription complex upstream of the initiation site. Clarification of the exact mechanism of transcriptional initiation for CNTF requires further study. So far, we have failed to demonstrate promoter activity by trans-

TABLE 1. Treatments used to investigate factors which alter CNTF mRNA levels in astroglial and Schwann cell cultures

Treatment	Concentration	Time	Effect on astrocytes	Schwann cell
γ-IFN	300 U/ml	72 h	3-fold ↑	No effect
forskolin	1–20 μM	24 h	↓	No effect
dd-FK	20 μM	24 h	No effect	
8-br-cAMP	1 mM	24 h	↓	No effect
dibu-cAMP	1 mM	24 h	↓	
bFGF	2–20 ng/ml	24 h	↓	No effect
kFGF	100 ng/ml	24 h	↓	
FGF-5	100 ng/ml	24 h	↓	
aFGF	20 ng/ml	24 h	↓	
Isoproterenol	10 μM	24 h	↓	
Norepinephrine	0.1–0.2 mM	24 h	↓	
0.1% FCS		5–48 h	No effect	
SC CM		24 h	No effect	
SC co-cult.		24 h	No effect	
Low density		24 h	No effect	
Cycloheximide	10 μg/ml	3–24 h	No effect	
Ionomycin	1 μg/ml	3–24 h	No effect	
Kainic acid	50 μM	3–24 h	No effect	
IL-1	10 U/ml	5–24 h	No effect	No effect
IL-1 + CHX		5 h	No effect	
IL-1 + Dex		5–24 h	No effect	
IL-1 + RA		24 h	No effect	
IL-6	100 U/ml	24 h	No effect	
T3	1 μM	5–48 h	No effect	
Dexamethasone	1 μM	24 h	No effect	
T3 + RA		4 h	No effect	
Retinoic acid	1 μM	24 h	No effect	
EGF	20 ng/ml	24 h	No effect	
PDGF	10 ng/ml	24 h	No effect	No effect
TGF-β1	2 ng/ml	24 h	No effect	No effect
IFN-α	20 U/ml	24 h	No effect	
IFN-β	20 U/ml	24 h	No effect	
Somatostatin	1 μM	3–24 h	No effect	
Bradykinin	10 μM	24 h	No effect	
CGRP	1 μM	24 h	No effect	
Glucagon	1 μg/ml	24 h	No effect	
Sub P	1 μM	3–24 h	No effect	
VIP	1 μM	24 h	No effect	
Met-enkephalin	1 μM	24 h	No effect	
Histamine	100 μM	24 h	No effect	

fection of CNTF promoter/CAT reporter gene constructs into astrocyte cells by several transfection methods. We assume that this is because only a subset (~10%) of astrocytes in culture express CNTF protein (Stöckli et al., 1991). Since the transfection efficiency that we achieved in transfection experiments was about 10%, transfected CNTF-expressing cells would represent only ~1% of all cells. It appears that the CNTF promoter may be weak, but the mRNA is very stable (half-life, ~6 h), thus accounting for the moderate levels of mRNA that can be detected in astrocyte cultures and sciatic nerve.

Since elevated cAMP levels can down-regulate CNTF mRNA expression, it might be supposed that this down-regulation is mediated through phosphorylation by protein kinase A of the DNA binding protein CREB (cyclic AMP response element binding protein) which acts at the CRE (cyclic AMP response element) consensus sequence (for reviews see Brindle and Montminy, 1992; Karin et al., 1989; Montminy et al., 1990) to turn off CNTF gene transcription. However, we found no evidence of a consensus CRE sequence within 2 kb upstream or 1 kb downstream of the CNTF transcriptional start-site. A consensus sequence for the binding of the transcription-regulatory complex AP-1 is found at position −72 in the human and rat genes. Whether this element is involved in regulation of the CNTF gene is as yet unclear. One possibility is that the transcription factor CREB or the related molecule CREM down-regulate CNTF transcription through the AP-1 site, since it has been shown recently that both CREB and CREM can compete in vitro with members of the Jun DNA binding protein family for binding to the AP-1 site and also that in vivo CREB and CREM can negatively regulate AP-1 site dependant Jun-mediated transcription activation of a reporter gene construct (Masquilier and Sassone-Corsi, 1992).

The regulation of expression of CNTF mRNA in cultures of rat astrocytes and Schwann cells was investigated since these cell-types have already been demonstrated to produce CNTF protein: astrocytes in culture (Lillien et al., 1988; Stöckli et al., 1991) and in vivo (Stöckli et al., 1991) and Schwann cells in culture (Meyer et al., 1992) and in vivo (Stöckli et al., 1991). CNTF mRNA has been demonstrated in cultures of rat astrocyte cells (Rudge et al., 1992; Stöckli et al., 1989). Primary Schwann cells and astrocytes were isolated from

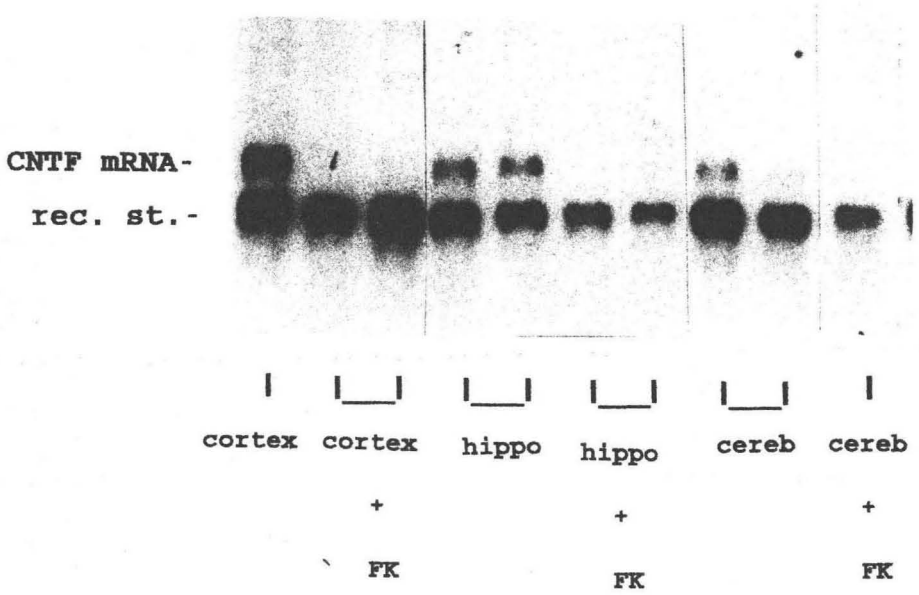


Fig. 4. Northern blot analysis of CNTF mRNA levels in 3-week-old astrocyte-enriched cultures from cells isolated from different brain regions, either untreated or treated for 24 h with forskolin (FK, 10 μM).

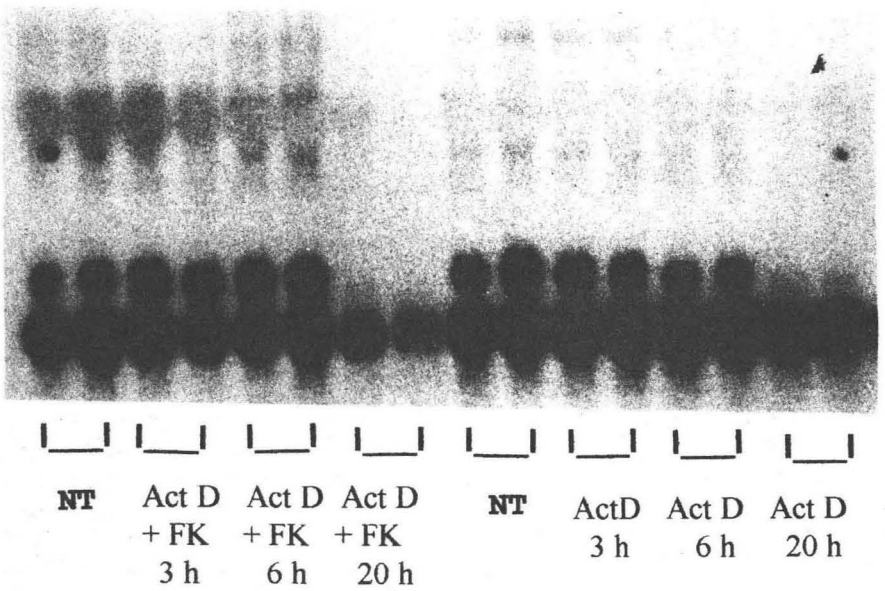


Fig. 5. Measurement of CNTF mRNA half-life in 3-week-old astrocyte-enriched cultures from new-born rat brain. NT, untreated cells; Act D, actinomycin D (10 μg/ml); FK, forskolin (10 μM).

new-born rat sciatic nerves and brain (where the levels of CNTF mRNA in vivo at this developmental stage are negligible) and grown in culture for 2–4 weeks.

Cultured Schwann cells, in contrast to the in vivo situation, expressed very low levels of CNTF mRNA and expression could not be increased by a variety of factors and growth conditions. We assume that the cells in culture do not follow their normal developmental programme which would be to begin to produce CNTF at about postnatal day four (Stöckli et al., 1991), or that specific in vivo requirements for CNTF expression are lacking when Schwann cells are grown in culture. Sendtner et al. (1992a), Friedman et al. (1992), and Seniuk et al. (1992) have shown that CNTF mRNA is also down-regulated in distal segments of the lesioned sciatic nerve, thus offering a plausible explanation for the lack of expression of CNTF mRNA in cultured Schwann cells, i.e., CNTF mRNA expression is correlated with intact nerve structure.

In contrast to the situation in Schwann cells, cul-

tured brain astrocytes express moderate levels of CNTF mRNA, although the levels in vivo in brain are relatively low, apart from the olfactory bulb and optic nerve (Stöckli et al., 1991).

CNTF mRNA can be down-regulated in astrocytes by forskolin, isoproterenol, norepinephrine, cAMP analogues, and by four members of the fibroblast growth factor (FGF) family. The physiological significance of these effects is unknown. Forskolin, norepinephrine, and isoproterenol are known to increase intracellular cyclic AMP levels: forskolin by activation of adenylate cyclase (Seamon et al., 1981), isoproterenol, and norepinephrine through β-adrenergic receptor activation (Atkinson and Minneman, 1991; Narumi et al., 1978). That the forskolin analogue dideoxyforskolin, which lacks the adenylate cyclase activation activity of forskolin (Laurenza et al., 1989), failed to lower CNTF mRNA levels is further evidence of a role for the cAMP second messenger pathway in the regulation of steady-state CNTF mRNA levels. The morphological changes induced by elevated intracellular cAMP levels in astrocytes, i.e., from polygonal to process-bearing cells, have been suggested to represent a more differentiated state (Kempski et al., 1987; Le Prince et al., 1991; Pollenz and McCarthy, 1986). This would fit with the down-regulation of CNTF mRNA by forskolin since in vivo CNTF mRNA levels are quite low in most brain regions (Stöckli et al., 1991). bFGF causes similar morphological changes in cultured astrocytes (Perraud et al., 1990 and references therein). However, there does not seem to be a direct correlation between intracellular cAMP levels, morphological transformation, and CNTF mRNA levels since some factors that elevate cAMP levels in astrocytes [VIP (van Calker et al., 1980), histamine (Agullo et al., 1990), and calcitonin gene-related protein (Lazar et al., 1991)] or cause morphological transformation [calcitonin gene-related protein (Lazar et al., 1991), calcium ionophores (Fawthrop and Evans, 1987), and PDGF (Besnard et al., 1987)] fail to down-regulate CNTF mRNA. bFGF also induces astrocyte proliferation in vitro (Pettman et al., 1985) and is thought to be involved in responses of astrocytes to brain injury (Baird and Walicke, 1989). Astrocytes in culture contain bFGF (Hatten et al., 1988; Woodward et al., 1992) but it appears not to be released and thus, as shown here, only exogenously applied bFGF can down-regulate CNTF mRNA. Whether bFGF regulates CNTF mRNA levels in vivo is unknown, but bFGF mRNA and protein levels are increased at the site of rat brain lesion (Finkelstein et al., 1988; Frautchy et al., 1991; Logan, 1990; Logan et al., 1992). Paradoxically, in view of the down-regulation of CNTF mRNA levels by bFGF in astrocyte cultures as shown here, CNTF mRNA levels are also increased at the site of brain lesion in cells which appear to be undergoing reactive gliosis (Ip et al., 1993).

The down-regulation of CNTF mRNA levels in astrocytes by bFGF is in contrast to the up-regulation of NGF mRNA levels by bFGF in the same cells (Spranger et al., 1990). Furthermore, several treatments which increase NGF mRNA levels in astrocytes, e.g., IL-1, TGF-α, EGF (Spranger et al., 1990), TGF-β1 (Lindholm et al., 1992), and phorbol ester (Zafra et al., 1992) had no effect on CNTF mRNA, which in addition to the differences in mRNA half-lives of CNTF (~6 h, this paper) and NGF (~90 min, Lindholm et al., 1988) and absence of secretion signal peptide in CNTF (Stöckli et al., 1989), adds further evidence to support the idea that there are fundamental differences in the physiological roles of these molecules.

Of the factors investigated, only IFN-γ elevated CNTF mRNA levels in astrocyte cultures. Indeed, in earlier experiments with human mixed spinal cord cultures, IFN-γ stimulated production of a cholinergic inducing activity from nonneuronal cells (Erkman et al., 1988). CNTF is an obvious candidate for such a factor. The cellular sources of IFN-γ are T lymphocytes and natural killer cells (Bancroft et al., 1987). IFN-γ is not normally found in the CNS but astrocytes (Wong et al., 1984), Schwann cells (Samuel et al., 1987), and oligodendrocytes (Bergsteinsdottir et al., 1992) in culture can be induced to express MHC class II antigens by IFN-γ treatment and adult human astrocytes are induced in vitro by IFN-γ to proliferate (Yong et al., 1991). Furthermore injection of IFN-γ into adult mouse brain promotes reactive gliosis at the injection site (Yong et al., 1991). It is hypothesised that T cell-derived IFN-γ may play a role in some pathological situations where there is access of cells of the immune system to the CNS (for review see Olsson, 1992; Wekerle et al., 1986). Elevation of CNTF expression by IFN-γ in astrocytes may be part of this response.

Further experiments using transgenic mouse models are in progress to elucidate the mechanism of regulation of CNTF expression in vivo.

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