A Bidirectional Regulation between the TR2/TR4 Orphan Receptors (TR2/TR4) and the Ciliary Neurotrophic Factor (CNTF) Signaling Pathway*

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Previously, we reported that the nuclear orphan receptor TR4 could induce transcriptional activity via the 5th intron of the ciliary neurotrophic factor (CNTF) α receptor gene (CNTFR-I5). Here we show CNTF could increase TR4 expression and enhance the DNA-binding capacity of TR4. Interestingly, the expression of TR2, a close family member of TR4, could also be induced by CNTF. In return, TR2 induced CNTFRα transcriptional activity through binding to a direct repeat response element of AGGTCTA within CNTFR-I5. The possibility of this mutual influence between TR2 and the CNTF signaling was further strengthened by in situ hybridization. Similar expression patterns of TR2 and CNTFRα were observed in most of the developing neural structures such as the ganglia, neural epithelia, spinal cord, and the periventricular areas of brain. Together, our data suggest that an interaction between TR2/TR4 and the CNTF signaling pathway may occur, supporting the hypothesis that TR2/TR4 may play important roles in neurogenesis.

The testicular receptor 2 (TR2)1 and testicular receptor 4 (TR4) orphan receptors belong to the same subfamily within the superfamily of steroid/thyroid hormone receptors (1, 2). They are termed “orphan receptor” because no ligands have been identified. As are other steroid receptors, TR2 and TR4 are transcriptional factors that trigger regulation of their target genes by binding to the hormone-response elements, thus leading to the activation of gene transcription (3). The hormone response elements for TR2 and TR4 consist of the AGGTCTA direct repeat with a 1–6-base pair spacing (DR1–DR6). The affinity of TR2 for these DRs follows the order: DR1 > DR2 > DR3, DR4, DR6 > DR3 (4); a similar tendency was observed for TR4.2 The finding of identical P and D boxes in the DNA-binding domains for TR2 and TR4 reasonably explains such a similar DNA-binding preference and implies that the function of these two receptors may overlap in certain biological processes. Indeed, similar actions of TR2 and TR4 were observed in the retinoic acid (RA) signal transduction pathway and in the Simian virus 40 promoter recognition3 (4–6). Earlier studies of TR2 function focused on its role in the reproductive organs where TR2 cDNAs were initially identified. Several TR2 isoforms, TR2–5, -7, -9, and -11, with different truncations in the C-terminal region, were isolated from testis and prostate cDNA libraries (7). High TR2 mRNA levels were detected in mouse embryos beginning at embryonic day 9 (E9) and in adult testis (8). Overexpression of TR2 causes aggressive fighting behavior observed in both male and female transgenic mice,3 suggesting TR2 may make important contributions to nervous system development. High abundance of TR4 messages found in many brain regions, such as the hippocampus and cerebellum (2), and within the active proliferating zone of the developing nervous system in embryos (9), has suggested that TR4 participates in neurogenesis.

Signal transduction of the ciliary neurotrophic factor (CNTF) requires binding to its receptor CNTFRα (10), which then sequentially associates with two structurally related β signal-transducing receptor components, gp130 and the leukemia inhibitory factor receptor β (LIFRβ) (11, 12). This association is then followed by signal transduction (11). Whereas gp130 and LIFRβ are ubiquitously distributed, CNTFRα expression is restricted to CNTF-responding cells of the nervous system (13). Interestingly, CNTFRα does not merely act as a receptor; a soluble form of CNTFRα can potentially interact with LIFRβ following its release from the membrane (14). Mice with null mutation in CNTFRα gene died shortly after birth and exhibited profound deficits in all motor neuron populations examined (15), suggesting that CNTFRα is essential for the developing nervous system.

The regulation of the CNTFRα gene expression remains unclear. A previous report published by Valenzuela et al. (16) showed that the fifth intron of the CNTF α receptor gene (CNTFR-I5) contains six copies of AGGTCTA-like sequences, which are preferentially bound by RA receptors, retinoid X

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1 The abbreviations used are: TR2, TR2 orphan receptor; mTR2, mouse TR2; TR4, TR4 orphan receptor; mTR4, mouse TR4; CNTF, ciliary neurotrophic factor; CNTFR-I5, 5th intron of the CNTF α receptor gene; DR1, direct repeat with one base pair spacing; E9, embryonic day 9; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; RA, retinoic acid; NBRE, TR3/NGFI-B/nur77 response element; LIFRβ, leukemia inhibitory factor receptor β, kb, kilobase(s); RACE, rapid amplification of cDNA ends; GFAP, glial fibrillary acidic protein.

2 Lee, Y.-F., Young, W.-J., Burbach, J. P., and Chang, C. (1998) J. Biol. Chem. 273, in press.

3 W.-J. Young, and C. Chang, manuscript in preparation.
receptors, and many orphan receptors (3, 4). These AGGTCA-like sequences accumulated in a 176-base pair small intron, including a direct repeat of the AGGTCA sequence with one base pair spacing (DR1) and one consensus NBRE sequence (17). The sequences of both DR1 and NBRE response elements are conserved among many species (9). When CNTFR-I5 was present in a reporter gene construct, it could enhance the transcriptional activity in the presence of TR4, suggesting that TR4 may be involved in the regulation of CNTFRα gene expression (9). We further hypothesized that TR2 and/or TR4 may be regulated by CNTF. Thus, a two-way interaction could result with CNTF to regulating TR2/TR4 expression and also TR2/TR4 to regulating CNTFRα expression.

CNTF is a neurocytokine that promotes the survival and differentiation of a variety of neuron cell types, including motor, sensory, sympathetic, parasympathetic, cerebellar, and hippocampal neurons (18); it also inhibits apoptotic cell death of cultured oligodendrocytes (19). Exogenous CNTF effectively blunts the progression of motoneuropathy (20, 21). Clinical trials of the CNTF application have been launched to treat patients with amyotrophic lateral sclerosis (ALS), a degenerative human motoneuropathy (22). Whereas CNTF may have important clinical significance, the mechanism of CNTF in neuron protection is not fully understood.

In the present study, we used P19 cells as a model system because these cells can be induced into the neuronal differentiation pathway in the presence of inducers, such as CNTF and RA (23, 24), and at a high cell density. Interestingly, the pattern of responsiveness to inducers appeared to be specifically influenced by cell-cell interactions: astrocytes showed greatly influenced by cell-cell interactions; astrocytes showed greatly enhanced differentiation in three-dimensional aggregating cell cultures, whereas in monolayer cell cultures a predominantly mitogenic response has been observed (24). As our preliminary data indicated, P19 cells endogenously express TR2, TR4, and CNTFRα (23). Therefore, we can test the effect of CNTF on the expression of TR2 and TR4 during neuronal differentiation. Here, our results show that CNTF treatment increased the population of TR2 and TR4 in P19 cells, and the DNA-binding ability of TR2/TR4 was enhanced. Such an increase may result in the induction of the enhancer activity of CNTFR-I5 and contribute to the expression of the CNTFRα gene. In addition, the distribution pattern of TR2 in the developing neural tissues correlated well with the pattern of CNTFRα reported by Ip et al. (13), supporting the physiological significance of our finding.

MATERIALS AND METHODS

Cell Culture Procedures—Differentiation of P19 cells was carried out as described previously (24) with minor modification. Briefly, cells in exponential growth were treated with trypsin-EDTA to remove them from the surface of Petri dishes and then plated at a density of 10^6 cells/ml into bacteria grade Petri dishes where they aggregated spontaneously and suspended. Cell aggregates were grown in α-MEM medium (Life Technologies, Inc.) supplemented with 10% charcoal-treated fetal calf serum and were allowed to sit for 3 days before treatment. The medium was replaced every 3 days. To induce neuron differentiation, rat CNTF (Boehringer Mannheim or American Research Products) at 20 ng/ml was added to the medium for 4 days. The aggregates were then plated into tissue culture dishes and examined morphologically 3 days later.

Immunofluorescence Assays—Aggregates were plated directly onto coverslips for staining in situ. After fixation in acetone for 5 min at −20 °C, the cells were incubated with the primary monoclonal antibodies at 10 μg/ml for glial fibrillar protein (GFAP) (PharMin- gen) or at a concentration of 10 μg/ml for glial fibrillar protein (GFAP) (PharMingen). This was followed by treatment with biotinylated secondary antibodies at 10 μg/ml (Vectastain Elite ABC universal kit; Vector Laboratories, Inc.) and then with fluorescein avidin (Vector Laboratories, Inc.). Cells were examined with an Olympic Photomicroscope equipped with epifluorescence optics. Photographs were taken by Kodak DCS System (Eastman Kodak Co.).

Cloning of the Mouse TR2 cDNAs—A positive mouse clone, named mTR2–1, which covers the nucleotide positions 832–1557, was isolated from an adult mouse testis agt11 cDNA library using the 1.7-kb EcoRI DNA fragment of hTR2–11 as a probe (7). The N and C termini were cloned by using RT-PCR (Perkin-Elmer) and 3′-RACE kits (CLONTECH), respectively. Reaction conditions followed the manufacturer protocols, and the total RNA purified from the adult mouse testis was used as a template. Oligonucleotides TR2–3 and TR2–8 were used to amplify the N terminus of the mTR2 cDNA. For 3′-RACE, the first strand of cDNA was synthesized from the testis RNA template with the C1(dt) primer in a reverse transcription reaction. A PCR reaction was then performed to amplify the C terminus of mTR2 cDNA using primers C1 (part of C1(dt) primer) and C2. The PCR products were cloned into pT7 Blue vector (Novagen) and sequenced using a Sequenase kit (U. S. Biochemical). The primers used are shown as follows: C1(dt), 5′-AAGATCCCGCATCGATTTTTTTTTTTTTTTTTT-3′; C1, 5′-AAAG-
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ATCCGTCGACATCGAT-3'; C2, 5'-TCCAGACTGCTGTTCTTATC-3'; TR2-3, 5'-TTTTGCAAGAGTGTCAAAT-3'; TR2-8, 5'-ATGGCAACCA-TAGAA-3'.

RNAScope Protection Assay—Total RNAs of P19 cells were purified by a standard CsCl centrifugation method, followed by extraction with phenol and chloroform. Riboprobes were labeled with [32P]UTP and in vitro transcribed (Ambion, Austin, TX) from plasmids containing a partial N-terminal fragment of mTR2 (mTR2A: nucleotides 1-250) (9). In each reaction, total RNAs (30 μg) were simultaneously hybridized with the antisense mTR2, mTR4, and β-actin riboprobes, using the HybSpeed Ribonuclease protection kit (Ambion). Procedures followed the protocols suggested by the manufacturer (Ambion). The protected bands were visualized and the intensity of each band was quantified using a Molecular Dynamics (Molecular Dynamics) PhosphorImager. Positions of each riboprobe transcript were confirmed using each sense and antisense riboprobes included in each batch of reactions.

Fold Induction

![Graph showing the induction of TR2 and TR4 transcripts during CNTF-induced neuron differentiation.](image)

**Fig. 2.** Induction of the TR2 and TR4 transcripts during CNTF-induced neuron differentiation. A, P19 cell aggregates were treated with CNTF (20 ng/ml) from 0 h to 3 days as described. Total RNA samples (30 μg/reaction) were simultaneously hybridized with various end-labeled riboprobes. Following hybridization, samples were digested with RNase H/T1 and loaded onto a urea-denaturing polyacrylamide gel. The expected sizes of protected bands for mTR2, mTR4, and β-actin are 81-, 250-, and 125-base pairs, respectively. The resulting autoradiograms were visualized by a PhosphorImager. Positions of each band are indicated at left. The sizes of RNA marker in nucleotides (lane M) are indicated at left. B, quantitative analysis of mRNA levels of mTR2 and mTR4 in response to CNTF treatment. All these data were quantified by the ImageQuant program (Molecular Dynamics). The individual level in the untreated control is counted as 1. The results from three independent experiments are expressed as mean ± S.D.

**RESULTS**

**Coupled in Vitro Transcription and Translation—**Plasmids pSPUCATK-T2 (5) and pcMX-TR4 (9) containing the full length of human TR2 cDNA and of TR4 cDNA, respectively, were in vitro transcribed and translated using the TNT system (Promega).

**Electrophoretic Mobility Shift Assay—**EMSA was performed as described previously (22) with minor modification. Briefly, the reaction was performed by incubating the [γ-32P]-end-labeled human CNTFR-DR1 probe (5'-GCCCTGACCTCTGACCTC3') (2 × 10^6 cpm/0.2 ng) with the Oct-1 normalized P19 cell nuclear extracts or 2 μl of in vitro translated protein. For antibody supershift assay, 1 μl of the monochlonal antibodies specific for TR2 or TR4 (9) were incubated with the reactions for 15 min at 25 °C prior to loading on a 5% native gel. After electrophoresis, the gel was dried and exposed overnight to a Kodak X-AR film.

**Scatchard Analysis—**The protein-DNA binding assay was performed as described previously (4). Briefly, 2 μl of in vitro translated TR2 protein was incubated with various concentrations of the [γ-32P]-end-labeled CNTFR-DR1 probe. Protein-DNA complexes were resolved on a 5% non-denaturing polyacrylamide gel at 4 °C. After exposing to an x-ray film, the respective bands of the free probe and the protected DNA-DNA complex were excised and counted directly in a scintillation counter (Beckman). The radioactivity ratio between the specific protein-DNA complex and the free DNA probe (bound/free) with respect to the radioactivity of specific DNA-protein complex (bound, nM) was plotted. The dissociation constant (Kd) and Bmax values were generated from the Edna program (Biosoft).

**Northern Blot Hybridization—**RNA samples (30 μg) were electrophoresed and transferred onto a nylon membrane (Amersham Pharmacia Biotech). The probe was labeled with [α-32P]dCTP using a random hexamer labeling kit (Amersham Pharmacia Biotech). The blot was hybridized with the mTR2N (nucleotide 1-282) probe. After stringent washing in 0.1× SSC solution at 55 °C for 15 min, the blot was exposed to an x-ray film (Kodak).

**In Situ Hybridization—**Embryos from C57BL/1J (Harlan Sprague-Dawley) were collected from E9 to E16. Section preparation and in situ hybridization were performed as described previously (4). To normalize the transcription efficiency, the p-galactosidase plasmid, pCMV5 (Clontech), was co-transfected. Results were plotted as mean ± S.D. of at least three independent experiments of CAT expression normalized to the p-galactosidase activity.

**Electrophoretic Mobility Shift Assay—**EMSA was performed as described previously (22) with minor modification. Briefly, the reaction was performed by incubating the [γ-32P]-end-labeled human CNTFR-DR1 probe (5'-GCCCTGACCTCTGACCTC3') (2 × 10^6 cpm/0.2 ng) with the Oct-1 normalized P19 cell nuclear extracts or 2 μl of in vitro translated protein. For antibody supershift assay, 1 μl of the monochlonal antibodies specific for TR2 or TR4 (9) were incubated with the reactions for 15 min at 25 °C prior to loading on a 5% native gel. After electrophoresis, the gel was dried and exposed overnight to a Kodak X-AR film.

**Transfection and CAT Assay—**Reporter plasmids, containing the CNTFR-I5 inserted into the pCAT-promoter vector (Promega), were as described previously (9). Chinese hamster ovary cells (CHO) were cultured and transfected by the calcium phosphate co-precipitation procedure as described previously (4). To normalize the transcription efficiency, the p-galactosidase plasmid, pCMV5 (Clontech), was co-transfected. Results were plotted as mean ± S.D. of at least three independent experiments of CAT expression normalized to the p-galactosidase activity.

**RESULTS**

**CNTF Stimulates Differentiation of P19 Cells—**To study the potential role of TR2 and TR4 in cells that follow a neuronal differentiation pathway, we first confirmed that P19 cells grown in a high cell density of an aggregation culture could be induced into differentiation following CNTF treatment. Differentiation of P19 cells into the glia cells and neuron cells were indicated by the presence of the GFAP and neurofilament (NF-L), respectively. As shown in Fig. 1, P19 cell aggregates without transfected contained undifferentiated embryonal carcinoma cells that resembled extracellular embryonic carcinoma and neither GFAP nor NF-L-containing filaments were observed (Fig. 1A and B). In contrast, approximately 10–15% of the CNTF-treated P19 cell aggregates developed processes and appeared to establish contacts with other cells. In addition to this morphological change, GFAP, the biochemical indicators for the glia cells, appeared in many CNTF-treated cells, and a minor...
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Increased TR2 and TR4 Expression Correlates with Early Neuronal Differentiation of P19 Cells as Induced by CNTF—The expression of TR2 and TR4 in P19 cell aggregates was examined during the course of CNTF treatment. As shown in Fig. 2, both TR2 and TR4 mRNA levels were increased 10-fold in P19 cell aggregates after 2 days of CNTF incubation but then declined as differentiation progressed. The basal expression level of TR2 mRNA is low such that multiple exposures are required for visualization of the TR2 band in the P19 RNA sample without the CNTF treatment (t = 0).

CNTF Enhances TR4 Protein Binding to CNTFR-DR1—As DNA-binding is essential for TR2 and TR4 to carry out their transcriptional activity, we developed an EMSA to examine the DNA-binding activity of TR4, using the CNTFR-DR1 as a probe. Crude nuclear extracts were isolated from the CNTF-treated P19 cell aggregates, and the endogenous TR4 protein was separated from other DR1-binding proteins by the TR4-specific monoclonal antibody, which supershifts the TR4 protein-DNA complex (Fig. 3). RA-treated aggregated P19 cells served as a positive control. As shown in Fig. 3A, the amount of TR4-CNTFR-DR1 complex (solid arrow) was low in undifferentiated P19 cells (lane 5) and was increased 80-fold upon CNTF treatment (lane 6). Interestingly, CNTF treatment resulted in TR4 being the predominant DR1-binding factor (>50%) in P19 cells (Fig. 3C). However, when the TR4 protein levels were examined by a Western blotting analysis (Fig. 3B), only a 6-fold increase was observed in the CNTF-treated nuclear extract. The discrepancy between the TR4 DNA-binding capacity and the immunoreactivity implies that posttranslational modification may be involved in activating TR4 for DNA-binding. Another possibility could be the suppression of other DR1-binding proteins by CNTF treatment, or the discrepancy observed is just a coupling efficiency difference. Consistent with the idea that RA has a broad effect on protein expression, we showed that the TR4 protein level was dramatically increased by the RA treatment (Fig. 3B); however, the expression of other DR1 binding proteins were proportionally increased by RA as well (Fig. 3A). Thus, TR4 represents 25% of all populations binding to CNTFR-DR1 (Fig. 3C). These data suggest that the activity of TR4 may dominate over other DR1-binding proteins in response to CNTF and could potentially play a major role in mediating the CNTF action.

TR2 Binds to CNTFR-DR1 with High Affinity—EMSA was performed with in vitro translated TR2 using the 32P-labeled CNTFR-DR1 oligonucleotide as a probe. As shown in Fig. 4A, a specific DNA-protein complex was formed in the presence of both probe and TR2 (lane 3, solid arrow) but was absent in the reaction containing the probe only and in the mock-translated control (lane 2). This TR2-CNTFR-DR1 complex could be abolished by a 10- or 100-fold molar excess of unlabeled CNTFR-DR1 oligonucleotide (lanes 4 and 5), but remained intact in the presence of a 100-fold molar excess of mutant CNTFR-DR1 oligonucleotide (lane 6). Moreover, this retarded complex could be fully supershifted in the presence of the anti-TR2 monoclonal antibody (lane 8, open arrow). As a negative control, an unrelated monoclonal antibody with the same subtype (IgM) showed no effect on the retarded complex (lane 7). Together, these data indicate that CNTFR-DR1 is a specific binding site for TR2.

population showed NF-L immunoreactivity (Fig. 1C and D).
To determine the binding affinity of TR2 and CNTFR-DR1, we performed the Scatchard analysis by EMSA. The typical EMSA pattern of protein-DNA complex formed between increasing amounts of the CNTFR-DR1 probe (0.0039–1 ng) and fixed amounts of TR2 was shown in Fig. 4B. The radioactivity DNA complex and the supershift band are indicated by the solid and hollow arrows, respectively. B, the binding profile of in vitro expressed TR2 to CNTFR-DR1 was resolved by EMSA. In binding reactions, a constant amount of TR2 was incubated with varying concentrations of the labeled CNTFR-DR1 probe, as indicated. When the probe amount was limited, only the TR2 protein-DNA complex was seen (lanes 2, 3, and 4), indicating strong affinity of TR2 over the CNTFR-DR1 probe.
FIG. 6. Sequencing and specificity analyses of the mTR2 cDNA fragments. A, alignment of the nucleotide and the deduced amino acid sequences of mTR2 and mTR4. These sequences are numbered on the left. The mTR4 residues that are identical to the mTR2s are shown by hyphens (-). Gaps were introduced to obtain an optimal match. The missing residues are shown by asterisks (*). The putative DNA-binding domain.
of the specific complex (bound) and unbound (free) probe was quantified for the subsequent Scatchard plot analysis. The results are consistent with a single binding population for the specific DNA-protein complex with a dissociation constant ($K_d$) of 0.039 nM and a $B_{\text{max}}$ of 0.3 nM. This binding affinity is about 2-fold higher than that of TR4 (9) and about 30–90-fold higher than the $K_d$ range for other steroid receptors and their response elements (26). At very low probe concentrations, the specific protein-DNA complex was still visible but not the free probe (Fig. 4B, lanes 2–4). This is consistent with the calculated dissociation constant.

The Enhancer Activity of CNTFR-I5 Was Induced by TR2 in a Dosage-responsive Manner—We examined whether TR2, like TR4, could activate transcriptional activity through CNTFR-I5. Various CAT reporter plasmids, containing CNTFR-I5 in different orientations and positions relative to the CAT gene (Fig. 5B), were co-transfected with TR2 expression plasmids into CHO cells. It showed that TR2 could induce CAT activity from 8–30-fold (Fig. 5C). In contrast, induction did not occur when either the antisense TR2 or backbone plasmids were transfected. Different orientations or positions of CNTFR-I5 did not appear to significantly affect TR2-mediated transcriptional activity. These results suggest that TR2 may contribute to the CNTFRα gene expression through the CNTFR-I5 enhancer.

Cloning the mTR2 cDNAs—The full-length and partial mTR2 cDNA fragments were cloned for studying the distribution of TR2 mRNA. DNA sequence analysis revealed that the full-length mTR2 consists of an open reading frame (590 amino acids) which is highly homologous to human TR2–11 (1). Sequence comparison between mTR2 and mTR4 (Fig. 6A) showed that the N terminus is the most divergent (30% homology), whereas the DNA-binding domain remains conserved (75% homology). Thus, we chose the N-terminal domain of mTR2 as a probe and tested its specificity by Northern blot hybridization. As shown in Fig. 6B, the mTR2N probe hybridized two bands in adult mouse testis with the sizes of 2.4 and 2.9-kb (lane 1, Fig. 6B). These transcripts were clearly distinct from those encoding mTR4, which are 7.8-kb (major band) and 2.8 kbp (minor band) (9). No TR2 signal was detected in kidney (Fig. 6, lane 2). These data suggest that the mTR2N probe is specific for TR2.

Expression of TR2 in the Developing Central and Peripheral Nervous System—To determine the tissue distribution of TR2 mRNA in mouse embryos, we performed in situ hybridization. Our results showed that TR2 transcripts were most prominent in developing neural structures (Fig. 7). During E11–E16, the TR2 signals were progressively restricted to the periventricular zones of developing brain vesicles, where many cells are in the mitotic cycle (Fig. 7, A and B) (27). Strong TR2 signals were found within the developing spinal motor neurons (Fig. 7A) and within the brain areas, such as cerebella (Fig. 7B), neocortex, striatum, and olfactory bulb (Fig. 7E). No signal was ever detected with the sense riboprobe (data not shown). Prominent TR2 expression was also detected in the peripheral neural tissues; ganglia with strong TR2 signal include the sympathetic (Fig. 7C, s), parasympathetic (Fig. 7D, X), and sensory ganglia, e.g. the dorsal root (Fig. 7, B and C, drg) and trigeminal ganglia (Fig. 7D, V). In addition, TR2 transcripts were abundant in targets of sensory innervation, such as the developing neural epithelia of the inner ear, nasal cavity, tongue, and retina (Fig. 7, A, D, and F). TR2’s localized expression is consistent with its potential participation in neurogenesis, especially during the events of early neuron proliferation or differentiation.

DISCUSSION

The Effects of CNTF on P19 Cell Differentiation and on the Expression of TR2 and TR4—Previous studies have shown that CNTF has a potent effect on the survival and differentiation of P19 cells (23). According to Gupta et al. (23), around 10–15% of P19 cells with CNTF treatment developed neurites. These process-bearing cells expressed the neuronal markers HNK-1 and neurofilament as well as the carbohydrate marker of neuronal differentiation. In addition, we observed CNTF-treated P19 cells developed into glia cells that grew neurites and expressed the GFAP marker. However, the neuronal marker NF-L appeared in a population that is morphologically different from the cells expressing GFAP. Recently, Bonni et al. (27) reported that CNTF triggers the differentiation of the cortical precursors into astrocytes, indicating the gliogenetic effect of CNTF on P19 cells is similar to that on the cerebral cortical precursors in certain ways. Thus, P19 cells could be a suitable model for our study.

The mechanism by which CNTF induces gene transcription has been well studied (27, 28). Activation of the CNTF receptors stimulates JAK-kinases to phosphorylate STAT1 and STAT3, which belong to the family of signal transducers and activators of transcription (STAT). Phosphorylated STAT proteins then translocate to the nucleus and bind to the CNTF-response element (TTCCNNNAA or TTCCNNNNAA), leading to the activation of genes containing this DNA-element within their promoter, for example the tis11 and SOD1 (29, 30). In reporter gene assays, two copies of the CNTF response element are sufficient to confer rapid CNTF responsiveness and result in an 8.5-fold induction of transcription (28). Interestingly, we found four putative CNTF response elements in the human TR2 promoter (2.7 kb in size) (31). The study of the human TR2 promoter in relation to CNTF treatment with STAT1 cotransfection is under way. These findings reasonably explain the inducibility of TR2 upon CNTF treatment. Although the TR4 promoter has not been characterized completely, we predict it...
may contain a similar response element given the rapid mRNA and protein induction of TR4 following CNTF exposure (Figs. 2 and 3).

To the authors’ knowledge, steroid receptors induced by CNTF treatment have not been previously reported. Our finding that induction of TR2 and TR4 by CNTF treatment establishes the first evidence for steroid receptors cross-talk to the CNTF signaling pathway.

**CNTF Promotes the DNA-binding Capacity of TR4**—Our data show that the amount of TR4 protein binding to the CNTFR-DR1 increases dramatically after CNTF treatment (Fig. 3). Possible explanations are that the TR4 expression level was increased, or the DNA-binding affinity of TR4 toward CNTFR-DR1 was increased, or both. Our data showed discrepancy between the TR4 DNA binding capacity and TR4 immuno-reactivity, suggesting that protein modification such as phosphorylation, may be involved in the activation of TR4. An alternative explanation is that such a discrepancy could be because of a coupling efficiency difference. Correspondingly, TR2 has been shown to be activated via the cAMP-mediated phosphorylation induced by the neurotransmitter dopamine (32). The effects of CNTF upon TR2/TR4 signaling may provide clues to the mechanism of CNTF actions in neuron protection. Whether the CNTF pathway could cross-talk to the dopamine pathway mediated by TR2/TR4 is an intriguing question to ask.

**Comparison of the Expression Patterns for TR2, TR4, and CNTFRalpha during Embryogenesis**—Whereas the overall expression patterns of TR2 and TR4 are very similar during development, the specificity of each probe has been confirmed by Northern blot analysis. Both TR2 and TR4 were strongly expressed in the actively proliferating cell populations of brain and of many peripheral organs. Such a wide but cell type-specific expression pattern leads to the hypothesis that both TR2 and TR4 may be involved in a stage-specific instead of a tissue-specific process. As the TR2 probe was derived from the N terminus, which is conserved in all the TR2 isoforms identified (7), this expression pattern may be caused by more than one TR2 isoform.

Certain developing tissues express only TR2 and not TR4, such as the developing rhombomeres, retina, lens, and brachial arches at E11 (Fig. 7A) as well as the vagal ganglia at E15 (Fig. 7D). Interestingly, strong TR2 signals were found in the junction of each rhombomere and branchial arch, suggesting a contribution to boundary segmentation or reinforcement of these repeating units. Reciprocal expression kinetics were observed between TR2 and TR4, implying TR2 is more important in the early developmental stage, whereas TR4 works during the late development and the maintenance of the nervous system.

Recently, different chromosomal locations have been mapped for human TR2 and TR4 to 12q22 and 3p24.3 (31), respectively. This rules out the possibility that TR2 and TR4 are isoforms transcribed from the same gene. Instead, their overlapping distribution patterns reflect functional conservation through evolution.

The TR2 expression overlaps profoundly with the expression of the CNTFRalpha gene (13) within the developing nervous system and many nonneural tissues. These three gene transcripts are
co-expressed in large amounts within 1) the periventricular brain regions; 2) the motor neuron-containing tissues such as the striatum, ventral spinal cord, and muscle; and 3) the ganglia of sensory, sympathetic, and parasympathetic origins. The co-localization of these transcripts suggests that interaction between TR2 and CNTFRα could be physiologically relevant.

**TR2Induces the Intronic Enhancer Activity of CNTFR-I5**—Although the contribution of CNTFTR-I5 to the CNTFRα gene expression remains unknown, we demonstrated that CNTFR-I5 could function as an enhancer in the presence of TR2/TR4 in a reporter gene assay. In fact, other DR1 binding proteins, such as RAR and RXR, and the AGGTCA half-site binder, such as TR3, could also induce the CNTFR-I5 enhancer activity in the same reporter assay. The possibility for these steroid receptors to regulate CNTFRα gene expression therefore may depend on the availability of these factors.

Upon binding to a DR1 sequence, TR2 could either induce gene transcription through CNTFTR-I5 or repress the RA-induced transcriptional activation through a similar DR1 response element present in the CRBPII promoter (4). The mechanism for such a discrepancy is still unclear, but evidence suggests the gene context could be critical in determining the consequence of protein binding (33). Thus, CNTFTR-I5 could function as an enhancer in the presence of protein binding (33). Therefore, CNTFTR-I5 may have important roles in neurogenesis.

In summary, our data suggest that a two-way interaction of TR2/TR4 and the CNTF signaling pathways may occur, which supports the hypothesis that TR2/TR4 may have important roles in neurogenesis.

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