Endocrine, *vol. 9, no. 1, 27–32, August 1998* 0969–711X/98/9:27–32/$9.50

Induction of an Intronic Enhancer of the Human Ciliary Neurotrophic Factor Receptor (CNTFRα) Gene by the TR3 Orphan Receptor

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A hormone response element, CNTFRα-NBRE (5'-AAAGGTCA-3') has been identified in the fifth intron of the α component of ciliary neurotrophic factor receptor gene (CNTFR-I5) for the human TR3 orphan receptor (TR3). A specific binding between in vitro expressed TR3 and CNTFRα-NBRE was demonstrated by using electrophoretic mobility shift assay. A reporter gene assay using chloramphenicol acetyltransferase (CAT) showed that CNTFR-I5 has an enhancer activity that could be induced by TR3 in a dose-dependent manner. This induction was significantly reduced in the absence of CNTFRα-NBRE. Together, these results indicate CNTFRα-NBRE is sufficient to mediate TR3 action in inducing the enhancer activity of CNTFR-I5. Our finding may, therefore, suggest CNTFRα is a target gene regulated by TR3 and expand the role of TR3 in the nervous system.

Key Words: TR3; CNTFRα; NBRE; intronic enhancer.

Abbreviations: CNTFRα, alpha component of the ciliary neurotrophic factor; CNTFR-I5, the fifth intron of the CNTFRα gene; TR3, TR3 orphan receptor; NBRE, TR3/NGFI-B/*nur77* response element; DR1, direct repeat with 1-bp spacing; SDS, sodium dodecyl sulfate; TNT, coupled in vitro transcription and translation; CHO, Chinese hamster ovary cell; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay.

Introduction

Members of the nuclear receptor superfamily are transcription factors that can bind to specific DNA sequences called hormone-response elements (HREs) and then regulate the expression of their target genes *(1)*. This superfamily includes receptors for steroid, thyroid, vitamin D, and a large number of orphan receptors whose cognate ligands are still unknown *(2)*. The HREs of nuclear receptors can then be grouped into three categories: first, the palindromic half-site AGAACA is preferred for the binding by the receptors for androgen *(3)*, glucocorticoid *(4)*, mineralocorticoid *(5)*, and progesterone *(6)*; second, the direct repeat, AGGTCA, with various spacing is preferentially recognized by the receptors for estrogen *(7)*, thyroid *(8)*, retinoic acid *(9)*, retinoid X *(10)*, vitamin D *(11)*, and many orphan receptors *(12)*; third, the single half-site of AGGTCA proceeded by two specific flanking nucleotides is favored by some members that bind as a monomer, such as the TR3 orphan receptor (TR3) *(13)*, steroidogenic factor 1 (SF-1) *(14)*, and the thyroid receptor (T_3R) *(15)*. It is generally accepted that a steroid receptor can mediate activation or inactivation of its target gene(s) through binding to the HREs located in the promoter or other regulatory regions.

TR3 (also known as NGFI-B or Nur-77) belongs to the nuclear receptor superfamily and is categorized as an orphan receptor, because its ligand has not yet been identified *(16,17)*. Previous studies suggest TR3 is an immediate early gene product rapidly induced by a variety of stimuli *(18)*. TR3 also plays important roles in the regulation of steroidogenic enzyme expression *(19)*, in the control of T-cell receptor-induced apoptosis *(20)*, and in the control of androgen- or etoposide-mediated cell death in prostate cells *(21)*.

The mechanism by which TR3 exerts its biological function remains largely unknown, but previous studies indicate that TR3 can either bind to a TR3/NGFI-B/nur77 response element (NBRE ; 5'-AAAGGTCA-3') as a monomer *(19)* or form a heterodimer with retinoid X receptor (RXR) on a retinoid response element *(22)*. Recently, a novel mechanism was proposed for TR3 action, in which TR3 forms a homodimer on the palindromic NurRE of the proopiomelanocortin (POMC) gene promoter *(23)*, suggesting multiple mechanisms for TR3 regulation of gene expression.

Ciliary neurotrophic factor (CNTF) is a member of the cytokine superfamily. It utilizes a three-component recep-

Received January 28, 1998; Revised April 8, 1998; Accepted April 8, 1998.
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tor system consisting of an extracellular CNTF binding protein, known as CNTFRα *(24)*, as well as two signal transducing β-receptor subunits, gpl30 and LIFRβ, which it shares with its cytokine relatives *(25,26)*. The expression of CNTFRα is mostly restricted to the neural tissues and is detected in all neurons that have been shown to respond to CNTF *(27)*. Clinically, CNTF effectively protects motor neuron degeneration in human Parkinson's disease *(28)*. Consistent with the CNTF effect on motor neurons, the essential function of CNTFRα gene has been shown in mice lacking CNTFRα, i.e., the mutant mice exhibit profound motor neuron deficits at birth *(29)*. Although the important function of CNTFRα is clear, the mechanism of the CNTFRα gene regulation remains unknown.

Based on the genomic organization and DNA sequence published by Valenzuela and coworkers, the fifth intron of the CNTFRα gene (CNTFR-I5) contains one AGGTCA direct repeat with 1-bp spacing (DR1) and several AGGTCA-like sequences, which include a typical TR3 response element (NBRE) *(30)*. It is amazing because so many AGGTCA-like sequences are simultaneously localized within a small intron only 276 bp in size. Therefore, we cloned CNTFR-I5 and focused on studying the interaction between TR3 and CNTFR-I5. Specific binding between TR3 protein and CNTFRα-NBRE sequence was proven by electrophoretic mobility shift assay (EMSA). The enhancer activity of CNTFR-I5 induced by TR3 was demonstrated using a reporter gene assay. Together, our results suggest TR3 may function as an inducer in the CNTFRα gene regulation, providing the first evidence for TR3 to crosstalk to the neurocytokine signaling pathway.

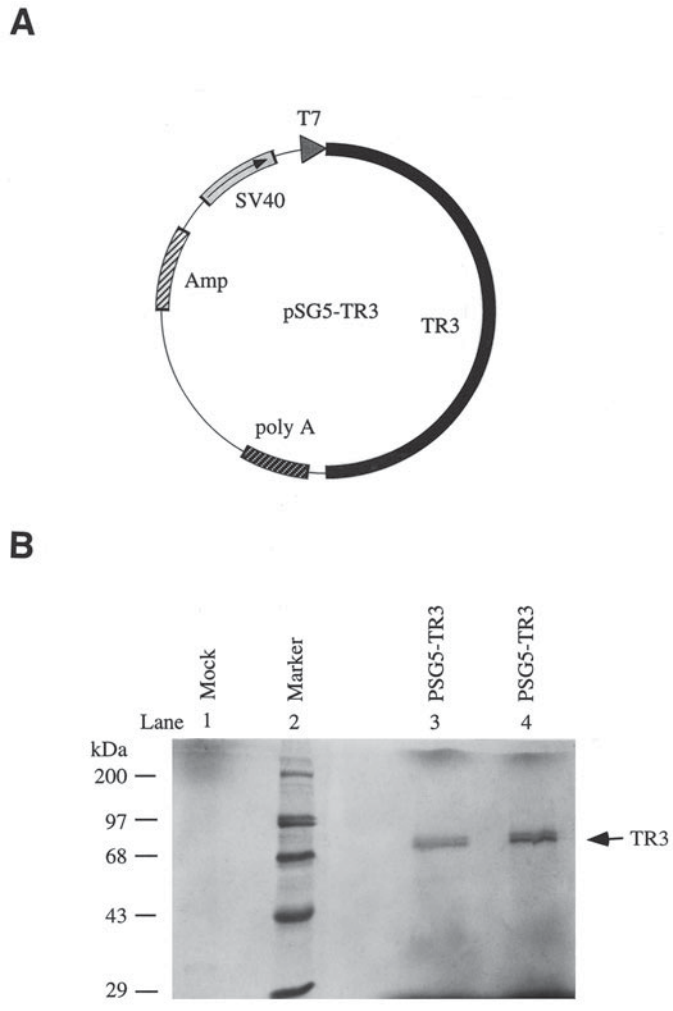

Fig. 1. Expression of the TR3 protein in vitro. **(A)** The map of the PSG5-TR3 plasmid. The coding region of the full-length TR3 cDNA was inserted into the pSG5 vector so that the TR3 expression could be under the T7 promotor control. **(B)** The expression of TR3 by TNT coupled reticulocyte lysate system. The products of the reaction products without (lane 1) or with (lanes 3 and 4) pSG5-TR3 plasmid were separated in 10% SDS-PAGE. The protein size markers (lane 2) were as shown. The TR3 protein with a predicted mol wt of 67 kDa is as indicated.

Results

In Vitro Expression of TR3 Protein

The pSG5-TR3 expression plasmid was constructed for in vitro expression. The plasmid map is shown in Fig. 1A. The mock-translated control produced no detectable product (Fig. 1B, lane 1), whereas the reaction containing PSG5-TR3 plasmids yielded a protein with the expected molecular mass of 67 kDa (Fig. 1B, lanes 3 and 4).

TR3 Binds Specifically to the CNTFRα-NBRE

To examine whether CNTFRα-NBRE could be a binding site for TR3, we carried out in vitro DNA binding experiments. Gel-retardation assays were performed with in vitro translated TR3 and the [^{32}P]-labeled CNTFRα-NBRE oligonucleotide as a probe. As shown in Fig. 2, a specific DNA protein complex was formed in the presence of both probe and TR3 (lane 4, hollow arrow), but was absent in the reaction containing probe alone (lane 1), probe with TR3-specific antibody (lane 2), or probe with the mock-translated control (lane 3). This TR3–CNTFRα–NBRE complex could be abolished by 10- to 100-fold molar excess of unlabeled CNTFRα-NBRE oligonucleotide (lanes 5 and 6), but remain intact in the presence of a 100-fold molar excess of "mutant" CNTFRα-NBRE oligonucleotide (lane 7). Moreover, this retarded complex could be fully supershifted in the presence of the anti-TR3 antibody (lane 8, solid arrow). Together, these data indicate that CNTFRα-NBRE is a specific binding site for TR3.

Enhancer Activity of CNTFR-I5 Induced by TR3

To investigate whether TR3 could possibly regulate the CNTFRα gene expression through interaction with CNTFR-I5, we carried out chloramphenicol acetyltransferase (CAT) assays with cotransfection of expression vectors and CAT reporter constructs into Chinese hamster ovary (CHO) cells. Three enhancer reporter plasmids were used in the present

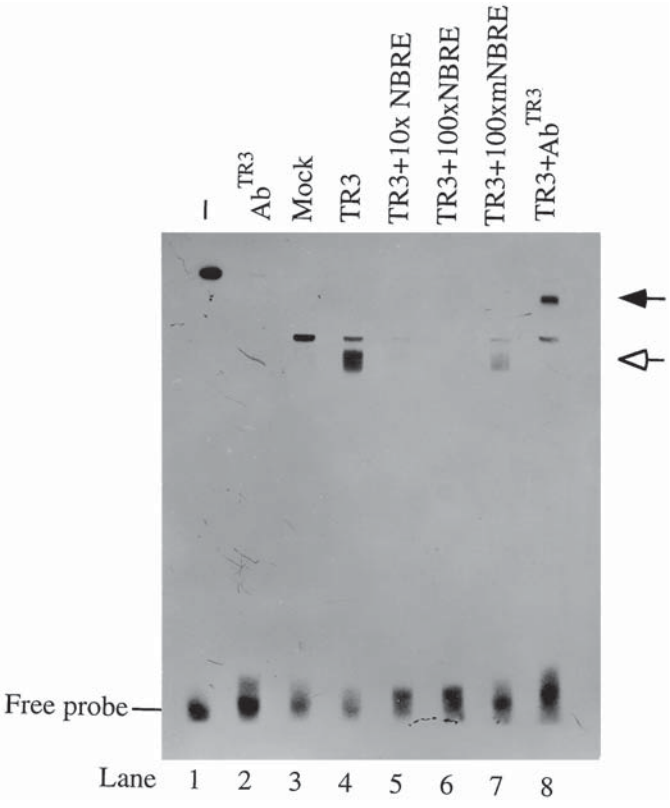

Fig. 2. Analysis of the binding of the in vitro expressed TR3 to the CNTFRα-NBRE by EMSA. TR3 was synthesized by TNT-coupled reticulocyte lysate system. The CNTFRα-NBRE oligonucleotides were ^{32}P-end-labeled and used as the probe in all the reactions. As negative controls, the binding reactions were carried out without lysate (lane 1), with lysate (lane 3), or with anti-TR3 antibody (lane 2). The binding profiles of the probe and the TR3 protein (lanes 4–8) in the presence of a 10-fold (lane 5) or 100-fold (lane 6) molar excess of unlabeled CNTFRα-NBRE, or a 100-fold (lane 7) molar excess of mutated unlabeled CNTFRα-NBRE. Supershift of the specific TR3–CNTFRα–NBRE complex was induced in the presence of anti-TR3 antibody (lane 8). The positions of the retarded complex and the supershift band are indicated by the hollow and solid arrows, respectively. The nonspecific band appears in the mock, and other samples (lanes 3–8) could be owing to the binding of an unknown factor present in the rabbit reticulocyte lysate to the probe. The spot appearing in the well (lane 1) is an artificial mark for the well position instead of a retarded band.

study to test whether the enhancer activity of CNTFR-I5 is position- or orientation-dependent (Fig. 3A). As shown in Fig. 3B, in the presence of the CNTFR-15, TR3 induces transcriptional activity up to 10- to 25-fold in a dose-dependent manner (compare lanes 3 and 4 to lane 2; lanes 9 and 10 to lane 8; lanes 15 and 16 to lane l4). In contrast, this induction did not occur when the antisense TR3 expression vectors were transfected (lanes 5, 6, 11, 12, 17, and 18). Different orientations or positions did not appear to affect the TR3-mediated transcriptional activity. These results suggest that TR3 could induce CNTFRα transcriptional activity via the enhancer of CNTFR-I5.

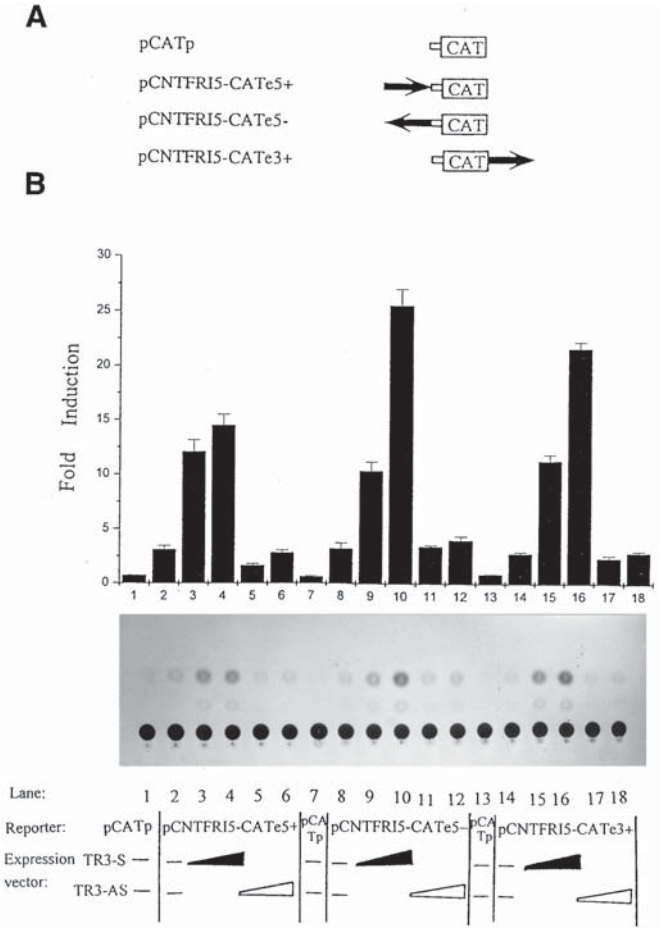

Fig. 3. Function analysis of CNTFRα-I5 gene fragment as regulated by TR3. **(A)** Construction of the CNTFR-I5-containing enhancer reporter plasmids. Names assigned to each construct are as indicated. CNTFR-I5 DNA fragments were inserted into either upstream (e"5") or downstream (e"3") of the vector pCAT-promotor (pCAT–). Relative orientations of enhancer fragments with respect to the promotor are indicated by arrows; and also indicated as "+" or as "–" for the same or opposite direction to the promotor, respectively. **(B)** Dosage-dependent induction of CAT activities by TR3 via CNTFR-I5. Various reporter constructs without (lanes 1, 2, 7, 8, 13, and 14) or with expression vector were transfected into CHO cells. The reporter constructs for transfection were either pCATp (lanes 1, 7, 13), CNTFRI5-CATe5 + (lane 2–6), or CNTFR-I5-CATe5- (lane 8–12), or CNTFR-I5-CATe3+ (lanes 14–18). Expression plasmids cotransfected include sense TR3 expression plasmid 0.5 μg (lanes 3, 9, and 15), 3 μg (lanes 4, 10, and 16), or antisense TR3 expression plasmid, 0.5 μg (lanes 5, 11, and 17), 3 μg (lanes 6, 12, and 18). Chloramphenicol conversion rates were calculated from phosphorImager quantifiable intensities. Fold induction was normalized relative to the CAT activity produced by the control plasmid pCATp without cotransfection with TR3 expression plasmid. The data are an average ±SD of at least three independent experiments.

Reduced TR3-Induced CNTFR-I5 Enhancer Activity in the Absence of CNTFRα-NBRE

To know whether CNTFRα-NBRE mediates TR3 action, we removed the NBRE sequence from the CNTFR-I5 containing reporter constructs. As shown in Fig. 4, the CAT

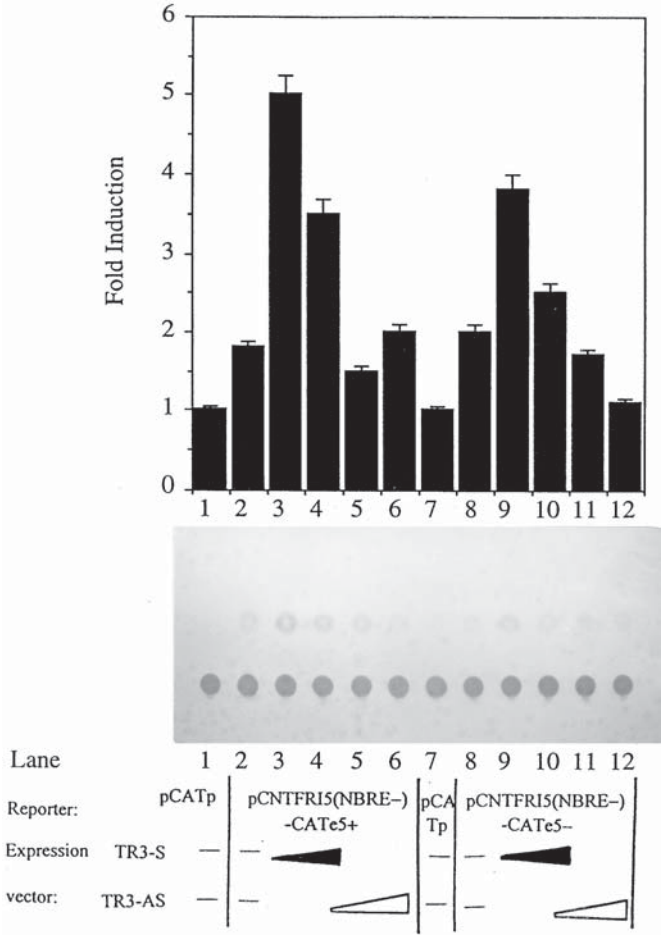

Fig. 4. The enhancer activity of the NBRE-deleted CNTFR-I5 induced by TR3. Various reporter constructs without (lanes 1, 2, 7, 8) or with expression vector were transfected into CHO cells, The reporter constructs for transfection were either pCATp (lanes 1 and 7) or CNTFR-I5-NBRE(–)-CATe5+ (lanes 2–6) or CNTFRI5-NBRE(–)-CATe5– (lanes 8–12). Expression plasmids cotransfected include sense TR3 expression plasmid, 0.5 μg (lanes 3 and 9), 3 μg (lanes 4 and 10), or antisense TR3 expression plasmid, 0.5 μg (lanes 5 and 11), 3 μg (lanes 6 and 12). Chloramphenicol conversion rates were calculated from phosphorImager quantifiable intensities. Fold induction was normalized relative to the CAT activity produced by the control plasmid pCATp without cotransfection with TR3 expression plasmid. Data are average of at least three independent experiments with the error bars representing ±SD.

assay results showed that CNTFR-I5 in the absence of the NBRE sequence has a much lower enhancer activity when induced by TR3, suggesting CNTFRα-NBRE mediates the major TR3 action in inducing the CNTFR-I5 enhancer activity.

Discussion

In the study of interaction between TR3 and CNTFR-I5, our data showed that TR3 could induce a high enhancer activity from CNTFR-I5 by binding to the CNTFRα-NBRE. In the absence of CNTFRα-NBRE, however, medium levels of induction remain. The possible explanations could be that TR3 can bind to other AGGTCA-like sequences with a lower affinity, TR3 may form heterodimers with the endogenous factors, such as RAR or RXR, on the AGGTCA direct repeat with 1-bp spacing (DR1) present in CNTFR-I5, or a synergistic effect happens through NBRE sites. In fact, our unpublished results showed that TR3 and RXR have a synergistic effect on the induction of CNTFRα gene expression, supporting such a possibility. In addition, many TR3-related proteins, such as Nurr1 *(31)*, NOR-1 *(32)*, NOR-2 *(32)*, MINOR *(33)*, and HZF-3 *(34)*, could also bind to NBRE specifically, which reasonably explains the high background we observed in the presence CNTFR-I5 without TR3 cotransfection. Whether these TR3-related proteins could contribute to the CNTFRα gene regulation remains unknown.

To know whether it is possible for TR3 regulation of CNTFRα gene expression to occur in vivo, we compared the expression patterns between TR3 and CNTFRα. It has been reported that TR3 has a widespread distribution in the central nervous system, with the highest level of expression in the cerebral cortex *(35)*. Moderate signals were detected in the hippocampus, certain nuclei of the hypothalamus, and the rhombencephalon. High levels of TR3 mRNA expression are also detected in the Purkinje layer and lateral deep nucleus of the cerebellum *(35)*. In response to various stimuli, the TR3 expression levels in the most of the brain areas were even higher *(36)*. According to the report published by Ip et al., CNTFRα is also widely expressed in the nervous system *(27)*. Thus, the expression patterns of CNTFRα and TR3 overlap to a great degree. This overlapping expression pattern and the active participation of TR3 in brain function further strengthen the possible physiological significance of our findings.

It has been shown that TR3 participates in its target gene regulation through binding to an NBRE or NurRE sequence. Thus far, four TR3 target genes, including CNTFRα, have been identified. By using cAMP as a secondary messenger, TR3 couples the signals derived from adrenocorticotrophic hormone, isoproterenol, and corticotropin-releasing factor to the activation of gene expression of 21-hydroxylase *(18)*, salivary gland-specific R15 protein *(37)*, and POMC *(23)*, respectively. Functional NBRE or NurRE sequences were identified in the promoter region of the genes. These data suggest that TR3 is an important mediator for hormonal and neurological responses in the hypothalamus–pituitary–adrenocortical axis. Although many stimuli are known to induce TR3 in a ligand-independent mechanism *(38,39)*, the natural ligand/activator that will activate TR3 for CNTFRα gene regulation remains unclear and will become an interesting topic for future study.

In summary, our novel finding that TR3 interacts with CNTFRα suggests that a crosstalk occurs between immediate early gene and the neurocytokine pathway, and that CNTFRα may represent the first neuron-specific target gene for TR3.

Materials and Methods

Cloning of the Fifth Intron of CNTFRα Gene

CNTFR-I5 was cloned by polymerase chain reaction (PCR) as previously described, using two oligonucleotides P1 (5'-CACCTTCAATGTGACTGTGC-3') and P4 (5'GTGCATGTAGCGAATGTGGC-3') flanking the CNTFR-I5 *(40)*.

The NBRE sequence was internally deleted from CNTFR-I5 using a PCR method. Typical PCR reaction was carried out with primers 1 and 2, as well as primers 3 and 4. Primers, P2 (5'-GGATCCCAAGGCAGGGCTGGGG-3') and P3 (5'-GGATCCTCAACCTCTGCCCCACCC-3'), are located in the flanking sequence of NBRE in CNTFR-I5. PCR products, P1/P2 and P3/P4, were obtained, with the expected sizes of 130 and 140 bp, respectively. These two PCR products were *Bam*HI-digested, ligated, and then cloned into the pT7 blue vector (Novagen) by a three-way ligation. DNA sequencing confirms the identity of the plasmids.

Reporter Gene Construction

The human CNTFR-I5 DNA fragments with NBRE or without NBRE sequences were inserted into the pCAT-promotor vector (Promega, Madison, WI) at either upstream or downstream sites relative to the CAT reporter gene. The plasmids pCNTFR-I5-CATe5+ and pCNTFR-I5-CATe5– *(41)* containing CNTFR-I5 with NBRE, as well as plasmids pCNTFR-I5(NBRE–)-CATe5+ and pCNTFR-I5(NBRE–)-CATe5-containing CNTFR-I5 without NBRE were inserted at the *Bgl*II site of pCAT-promotor vector. The plasmid pCNTFR-I5-CATe3+ has CNTFR-I5 inserted at the *Xba*I site of pCAT-promotor vector. "+" or "–' means the orientation of CNTFR-I5 is the same as or opposite to CAT expression, respectively.

In Vitro Expression of TR3 Protein

Plasmid PSG5-TR3 containing full-length human TR3 cDNA in the PSG5 vector (Stratagene, La Jolla, CA) was in vitro transcribed and translated by the rabbit reticulocyte lysate-TNT system (Promega), according to the manufacturer's instructions. The reaction products were analyzed by using 10% SDS-PAGE.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was carried out as described previously *(41)*. Briefly, 1 μL TNT-expressed TR3 was included in each binding reaction. Double strands of CNTFRα-NBRE (5'-GGCTTGACCTTTCCTC-3') were [γ-^{32}P] ATP end-labeled and used as a probe. Mutated CNTFRα-NBRE (5'-GGCTTCACCATTCCTC-3') contains two mutated nucleotides (letter underlined). For competition reactions, unlabeled double-stranded oligonucleotides were mixed with labeled probe before being adding to the reaction. For antibody supershift analysis, 1 μL of anti-TR3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated with the reaction for 15 min at room temperature before loading onto a 5 % native polyacrylamide gel.

Cell Culture, Transfection, and CAT Assay

CHO cells were cultured and transfected by the calcium phosphate coprecipitation procedures as described previously *(41)*. To normalize the transfection efficiency, the plasmid pCMV-β-galactosidase expression plasmid (Clontech) was cotransfected. The CAT assay was performed as described previously *(43)*. Results were plotted as the mean ± standard deviation of at least three independent experiments.

Acknowledgments

This work was supported by NIH grants DK 47258, CA 71570, and CA 68568. Win-Jing Young is supported by T32 CA 09363D NCI, NIH.

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