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Identification of a *cis*-acting element that interferes with thyroid hormone induction of the neurogranin (*NRGN*) gene

Beatriz Morte, Cruz Martínez de Arrieta, Jimena Manzano, Antonio Coloma, Juan Bernal*

Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM, Arturo Duperier 4, 28029 Madrid, Spain

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Abstract The neuron-specific RC3/neurogranin gene is regulated by thyroid hormone at the transcriptional level in brain and in cultured neuronal cells. Regulation in vivo displays exquisite regional selectivity which is not due to differential distribution of thyroid receptors and is most probably related to region-specific *trans*-acting elements. We have previously identified an intronic thyroid hormone responsive element in the human RC3 gene homolog, *NRGN*. In a search for *cis*-acting elements that might contribute to the specificity of thyroid regulation, we have identified a novel sequence, TTCCAAAATGG, which binds to a developmentally regulated protein, and interferes with T3 transactivation.

Key words: Thyroid hormone; Brain gene expression; Development; Nuclear receptor; Southwestern blotting

1. Introduction

Mammalian brain maturation is critically dependent on thyroid hormones (thyroxine, T4 and 3,5,3′-triiodothyronine, or T3). The active form of thyroid hormone, T3, binds to nuclear receptors [1], which are ligand-modulated transcription factors of the steroid hormone superfamily. T3 receptors (TR) bind target genes through specific DNA sequences known as T3 responsive elements (TRE). These elements usually recognize heterodimers between the TR and the 9-*cis* retinoic acid receptor, RXR [2]. In addition to RXR, other molecular entities participate in the action of T3, namely corepressors and coactivators which transduce the signal from the unliganded or liganded TR to the basal transcriptional machinery [3–5].

In brain, thyroid hormone coordinates the expression of target genes during development and in different regions, so that gene expression occurs in an orderly fashion during maturation [6,7]. One important question is how regional selectivity in thyroid hormone control of target genes is achieved. The problem can be illustrated from our previous studies of the control of the neuronal gene RC3/neurogranin [8]. Thyroid hormone regulates RC3 expression only in discrete regions, namely the caudate, the granular cell layer of the hippocampus, neocortical layer 6 and retrosplenial cortex. Other regions are insensitive, for example amygdala, upper layers of the cortex and pyramidal layer of the hippocampus, [9]. There is yet no explanation for this differential sensitivity of diverse neuronal populations. RC3 is a primary transcriptional response to T3 [10,11], and therefore additional cofactors are likely to be involved in the determination of the regional sensitivity to thyroid hormone. It is therefore of great interest to identify such putative cofactors since they likely act through modulation of the action of thyroid hormone and other epigenetic factors.

We have recently shown that the human RC3 homolog, *NRGN*, contains a TRE in the first intron [12]. This element was identified as a direct repeat of the consensus hexameric sequence AGGTCA separated by four nucleotides (DR4), as in other T3 regulated genes. This element was able to modulate the activity of a reporter gene under control of either homologous or heterologous promoters. In this paper we show that a sequence adjacent to the TRE binds a nuclear protein which interferes with T3 transactivation. Such a protein altogether with the *cis*-element where it binds to are likely involved in the regional specificity and/or timing of thyroid hormone action.

2. Materials and methods

2.1. Plasmids and oligonucleotides

The following plasmids were used in this study: pBLCAT-2, a chloramphenicol acetyl transferase (CAT) expression vector driven by the thymidine kinase (tk) promoter; a rat T3 receptor α-1 isoform in pCDM8, a gift from Dr. P.R. Larsen (Brigham and Women's Hospital, Boston, MA, USA); human RXRα expression vector in pSG-5, supplied by Dr. H. Stunnenberg (University of Nijmegen, The Netherlands). The oligonucleotides used for the gel retardation experiments were synthesized as two complementary strands with 5′ extensions. The TRE-containing oligonucleotides were synthesized according to the sequences of the NRGN TRE [12] and the TRE present in the long terminal repeat of the Moloney murine leukemia virus (Mo) [13], as follows (the half sites of the TREs are underlined):

NRGN TRE: 5′-TCGACTTCCAAAATGGGGATTAAATGAGGTAATATC-3′
Mo TRE: 5′-TCGACAGGGTCATTTCAGGTCCTTGC-3′
NR-Mo: 5′-TCGACTTCCAAAATGGGGATTGGGTCATTTCAGGTCCTTGC-3′

The mutated oligonucleotides were variants of the NRGN TRE with the indicated modifications (nucleotide substitutions are in italics):

M1: 5′-TCGACTTCCAAAATGG*TT*ATTAAATGAGGTAATATC-3′
M2: 5′-TCGACTTCCAAAATGGGGATTAAATGA*TT*TAATATC-3′
M4: 5′-TCGACG*ATCCAAGA*GGGATTAAATGAGGTAATATC-3′

*Corresponding author. Fax: (34)-91-5854587.
E-mail: jbernal@iib.uam.es

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And the mutated oligonucleotide of the NR-Mo:

M4-Mo: 5′-TCGACG*ATCCAAGA*GGGATTGGGTCATTTCA-GGTCCTTGC-3′

Non-specific oligonucleotide (NS):

NS: 5′-GATCCAAGAGTTTGACTGGCTGATTTCCAGTTT-GTG-3′

The oligonucleotide NS-x contains the TTCCAAAATGG sequence inserted in the NS sequence:

NS-x: 5′-GATCCAAGAGTTTGACTTCCAAAATGGCAGTT-TGTG-3′

For Southwestern blotting the double-stranded oligonucleotide 5′-AGCTTTCCAAAATGGTTCCAAAATGG-3′ was self ligated to prepare a probe containing tandem copies of the sequence.

For transactivation experiments, a single copy of each of the Mo TRE, NR-Mo and M4-Mo oligonucleotides was subcloned into the *Sal*I site of pBLCAT-2 vector.

2.2. Cell cultures and transient transfection assays

COS7 and N2a cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. GT1-7 cells were grown in modified Eagle's medium supplemented with 5% fetal bovine serum and 5% horse serum. Transfections and T3 treatment of the cultures were done as described in detail [10,14].

2.3. Isolation of nuclear extracts

Cells were harvested, resuspended in ice-cold hypotonic buffer (10 mM HEPES KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethanesulfonyl fluoride (PMSF)) incubated in ice for 10 min and centrifuged. The pellets were resuspended, incubated for another 20 min in ice in 50 ml of a hypertonic buffer (20 mM HEPES KOH pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF) and centrifuged. The supernatants were aliquoted and stored at −70°C. Rat brain nuclear extracts were prepared by resuspending purified nuclei in lysis buffer (400 mM KCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20 mM HEPES KOH pH 7.9, 0.5 mM DT, 0.2 mM PMSF, 2.5 mg/ml leupeptin, 10 mg/ml STI).

2.4. Electrophoretic mobility shift assays (EMSA)

TR and RXR were synthesized using the TNT® kit (Promega, TNT Sp6 Coupled Reticulocyte Lysate system). Double stranded oligonucleotides were labelled with T4 Polynucleotide Kinase and [γ-^{32}P]ATP, and purified with QIAquick Nucleotide Removal® kit (Qiagen). The EMSA procedure was exactly as described [12].

2.5. Southwestern blotting

Complementary oligonucleotides were annealed, phosphorylated, and ligated for 12 h at 15°C. The concatenated DNA (500 ng) was labelled by random priming with 50 μCi [α-^{32}P]dCTP. Nuclear extracts (50 μg protein) were boiled for 10 min in 6% SDS and 60 mM DTT and electrophoresed in a 5–10% polyacrylamide gradient gel in the presence of 10% SDS. The proteins were transferred to a nylon membrane (Immobilon; Millipore) in 20% methanol, 25 mM Tris, 0.2 M glycine by electrotransfer at 300 mA for 1 h. The filters were washed in binding buffer (40 mM HEPES pH 7.9, 200 mM KCl, 1 mM EDTA, 1 mM DTT) containing 6 M guanidine HCl, and then gradually renatured by five washes using serial two-fold dilutions of the guanidine HCl in binding buffer, with a final incubation in binding buffer. All washes were for 10 min at 4°C. The filters were blocked in 5% non-fat dry milk in binding buffer for 30 min at 4°C and then hybridized overnight at 4°C in binding buffer with 0.25% non-fat dry milk and 1×10^6 cpm/ml of the labelled probe. The unbound probe was washed away by incubation in the latter solution for 45 min at 4°C, and the blots were exposed to X-ray films for autoradiography.

3. Results and discussion

We have shown previously that the *NRGN* gene contains a

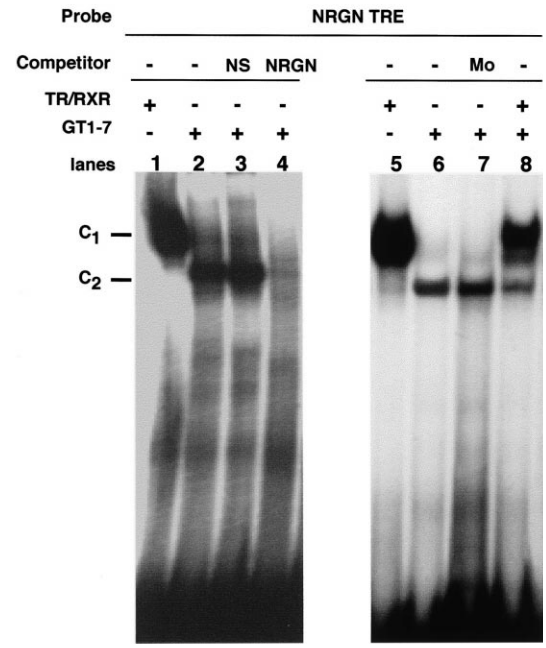

Fig. 1. EMSA demonstrating binding of nuclear proteins to the NRGN TRE oligonucleotide. The labelled NRGN TRE oligonucleotide was used as a probe. It was incubated with in vitro translated TR and RXR (lanes 1 and 5) or with nuclear extract from GT1-7 cells (lanes 2–4, 6 and 7) or a mixture of both (lane 8). C1 is the band produced in the presence of TR and RXR. C2 is the band produced in the presence of nuclear extract. NS: non-specific oligonucleotide. Mo: prototypical DR4 TRE present in the long terminal repeat of the Moloney murine leukemia virus.

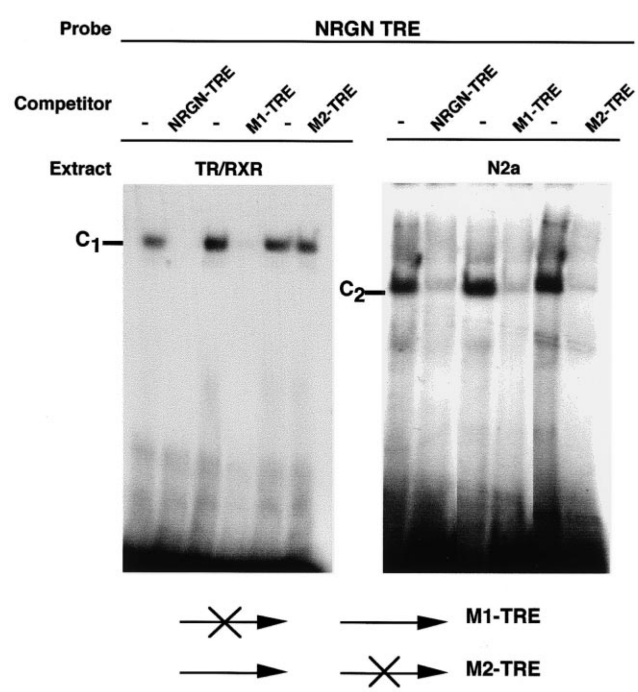

Fig. 2. Binding of nuclear proteins to NRGN TRE in the presence and absence of TRE half site mutants M1 and M2. A mutation in the second half site of the NRGN TRE abolished binding of TR/RXR but not of nuclear proteins.

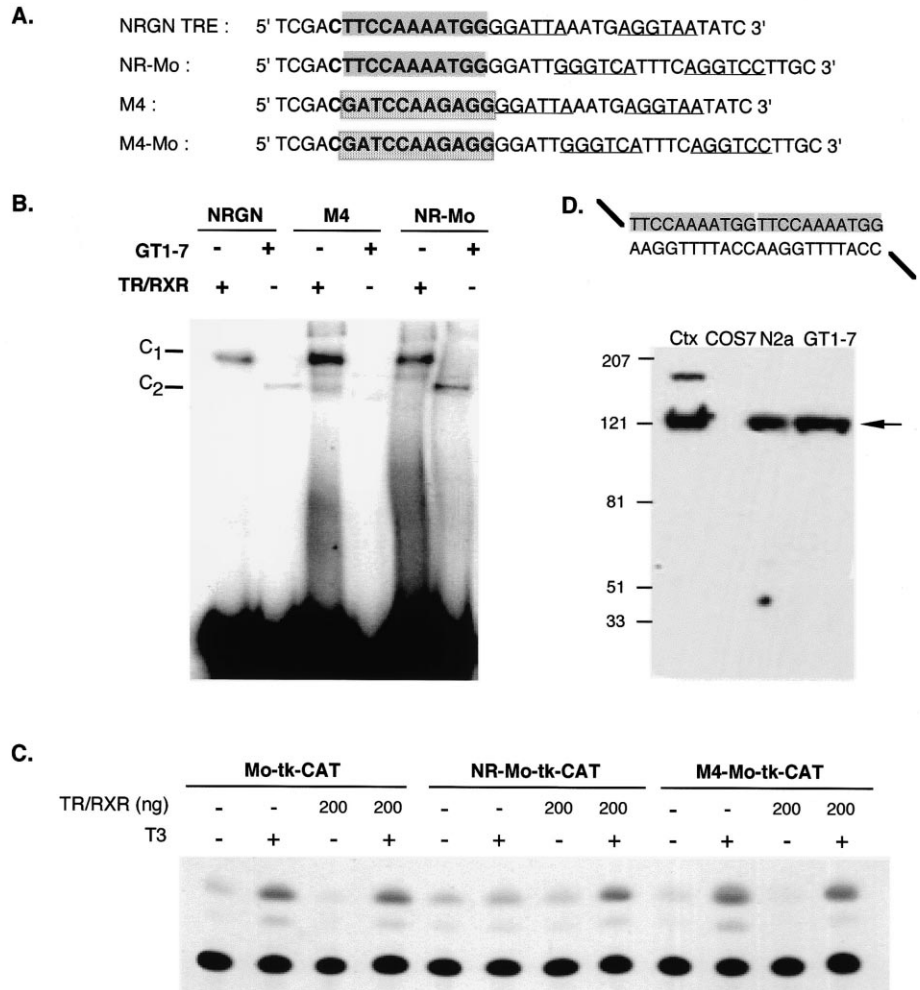

Fig. 3. A: Sequences of the oligonucleotides used in the experiments. B: EMSA using three of the oligonucleotides as probes, after incubation with the TR/RXR, or with GT1-7 nuclear extract. C: CAT reporter activity after transient transfection in GT1-7 cells. D: Southwestern analysis of nuclear proteins using the TTCCAAAATGG sequence as a probe.

functional TRE in the first intron [12]. Fig. 1 shows that using this element as a labelled probe, an electrophoretically retarded band (C1, lane 1) is produced when incubated with in vitro translated TR and RXR. However, when the same probe was incubated with nuclear extracts from hypothalamic, thyroid hormone-responsive GT1-7, a faster migrating band (C2, lane 2) was observed. This band was specific since it was not competed by excess of an unrelated sequence (lane 3), but was greatly reduced in the presence of an excess NRGN TRE oligonucleotide (lane 4). In contrast the C2 band was not obliterated by incubation with the strong TRE present in the Moloney murine leukemia virus LTR (Mo; Fig. 1, lane 7). Exactly the same results were obtained using extracts from the neuroblastoma cell line N2a. The result of these experiments suggested the presence of nuclear proteins in the cell extracts that bind specifically the NRGN TRE oligonucleotide with a different specificity than the TR-RXR heterodimer. Incubating the labelled NRGN TRE oligonucleotide with a mixture of both, TR/RXR and GT1-7 nuclear extract led to formation of two major shifted bands, corresponding to C1 and C2 (Fig. 1 lane 8). This result suggested that the oligonucleotide probe bound either the receptor heterodimer or the nuclear extract protein, since the mobility of the C1 and C2 bands was not changed, and no higher mobility complexes were observed.

To learn more about the binding specificity of both sets of proteins we set out experiments in which the labelled NRGN TRE was incubated with either TR/RXR or nuclear extracts in the absence or presence of competitors. As competitors we used the wild type or mutated versions of the NRGN TRE sequence, as shown in Fig. 2. In presence of TR/RXR, the heterodimeric band (C1) was obliterated by the wild type oligonucleotide, or by a mutated version (M1) containing a nucleotide substitution in the 5′ half site, but not by M2, which contains a mutation in the 3′ half site. These data are consistent with previous results [12] which showed the importance of the 5′ half site of the TRE. In contrast, formation of the C_2 complex (Fig. 2, right panel) took place in the presence of nuclear extracts, and was obliterated by either the NRGN TRE wild type, or by the mutated versions. These results strongly suggested that the nuclear protein did not bind to the DR4 element, but to the sequence TTCCAAAATGG, located 5′ to the TRE in the oligonucleotide used for the EMSA experiments.

We checked whether this sequence was able to bind nuclear proteins, and influence T3-mediated transactivation when

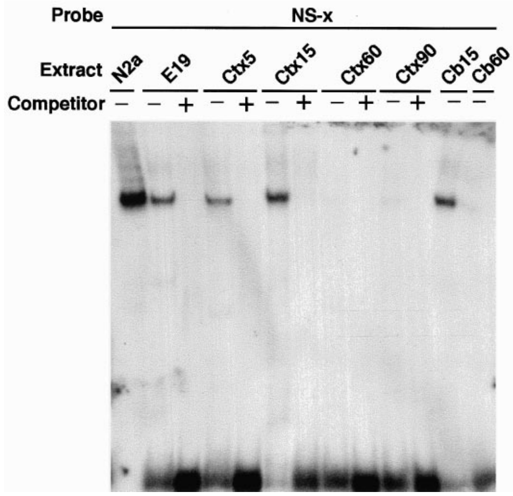

Fig. 4. EMSA using nuclear extracts from N2a cells, embryonal rat brain (E-19), cerebral cortex (Ctx) of postnatal days 5, 15, 60 and 90 and cerebellum of postnatal days 15 and 60. Competition assays were performed with a 200-fold molar excess of the unlabelled oligonucleotide.

placed adjacent to a strong TRE such as the Mo TRE (NR-Mo, Fig. 3A). Using the native NRGN oligonucleotide as a probe (Fig. 3B), a heterodimer band was again observed in the presence of in vitro translated TR/RXR (C_1), or a faster migrating band (C_2) in the presence of GT1-7 cell nuclear extract. The Mo TRE behaved similarly to NRGN when the TTCCAAAATGG sequence was placed adjacent to the DR4. In addition, when this sequence in the NRGN TRE native oligonucleotide was replaced by a different but closely related sequence (Fig. 3A, M4-NRGN) the heterodimer band was still present, obviously due to intact DR4, but the faster migrating C_2 band was absent.

As an attempt to functionally correlate binding with transactivation we used tk-CAT reporter vectors with either the native Mo TRE or the NR-Mo TRE (see Fig. 3A) located upstream from the tk promoter. These reporter constructs were used for transactivation assays in GT1-7 cells with or without expression vectors for TR and RXR. CAT activity was measured after incubating cells in the absence or presence of T3 (Fig. 3C). Since GT1-7 cells contain physiological amounts of functional TR and RXR [10], the cells transfected with the Mo-tk-CAT construct responded to the addition of T3 with an increased CAT activity. Addition of expression vectors encoding TR and RXR resulted, as expected, in a decreased basal expression of the reporter, due to the repressor activity of unliganded receptors, but not in higher T3 stimulation. In contrast, cells transfected with NR-Mo-tk-CAT did not respond to T3 unless cotransfected with exogenous receptors. The mutated version of this construct (M4-Mo) behaved as the Mo-tk-CAT construct. These data demonstrate that an endogenous nuclear protein binds to the TTCCAAAATGG sequence, then interfering with activation by endogenous RXR/TR receptors.

The molecular mass of the nuclear protein present in the C_2 complex was estimated by Southwestern analysis (Fig. 3D). Nuclear proteins were prepared from COS7, GT1-7 and N2a cells, fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nylon membrane. The protein blot was probed with a labelled oligonucleotide containing tandem copies of the TTCCAAAATGG sequence. After hybridization a single band of M_r 121 000 was identified in GT1-7 and in N2a, but not in COS7 nuclear protein extracts. A band with the same electrophoretic mobility was also present in nuclear extracts from cerebral cortex.

Presence of the C_2 complex varies during development, as it is revealed by analysis of nuclear proteins harvested from total embryonal brain (E-19) and from cerebral cortex and cerebellum from rats at different postnatal ages. The extracts were analyzed by EMSA (Fig. 4) after incubation with a labelled oligonucleotide containing the TTCCAAAATGG sequence (NS-x), either in the absence or in the presence of an excess unlabelled oligonucleotide. As expected, the shifted band produced using nuclear extracts from N2a cells showed up (Fig. 4). Nuclear extracts from rat brain produced a similar band with extracts from either E-19 embryos or cerebral cortex of postnatal days 5 and 15 (Ctx-5 and Ctx-15, respectively). The band was also produced with the postnatal day 15 cerebellum (Cb-15) but not with cerebral cortex or cerebellum from adult rats (Ctx-60, Ctx-90, Cb-60).

These experiments showed that the sequence TTCC-AAAATGG, present in the first intron of the human neurogranin gene, specifically binds a nuclear protein of M_r 121 000. Binding of this protein in a position which is adjacent to the TRE interferes with TR/RXR-dependent transactivation by T3. It is known that the extent of transactivation by T3 in cells overexpressing TR and RXR is very variable among different cell lines [15], suggesting that cell factors other than the receptors may limit the T3 response. These factors which may not necessarily interact with the T3 receptors, but with the target gene, are likely to modify the hormonal response [16–19]. The protein identified here shares most of the properties defined for such factors. Moreover, it is developmentally regulated, since it is more abundantly expressed in young animals than in adult animals. Recently COUP-TF, another developmentally regulated protein, has been shown to antagonize the action of T3 in embryonal and neonatal rat brain [20]. To precisely define the physiological role of the 121 000-M_r protein in NRGN expression and T3 regulation, cloning of this protein is currently in progress in our laboratory.

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