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# Variable RXR requirements for thyroid hormone responsiveness of endogenous genes

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**Abstract**

Thyroid hormone receptors heterodimerize with retinoid X receptors *in vitro* and it is widely assumed that these heterodimers mediate the T3 induction of target genes. However, the importance of RXR for the T3 induction of endogenous genes has not been assessed. We used cDNA microarrays to identify 54 genes induced by T3 in Neuro2a cells that express thyroid hormone receptor beta. RNA interference-mediated knock down of endogenous RXRs showed that these genes vary from being highly dependent on RXR for T3 induction to being independent of RXR. Thus, the availability of RXR may differentially regulate the T3 induction of subsets of genes within a cell. Furthermore, coregulatory proteins that preferentially interact with TR homodimers or RXR–TR heterodimers may further expand the range of T3 response for genes within the same cell.


*Keywords:* Retinoid X receptor; Thyroid hormone receptor; T3; Heterodimer; Homodimer

## 1. Introduction

Thyroid hormone receptors (TRs) and retinoid X receptors (RXRs) are members of the nuclear hormone receptor family of ligand activated transcription factors which regulate many important processes in development and physiology. Nuclear hormone receptors mediate gene induction through interactions with specific response elements often located in the promoter sequences of target genes. Nuclear receptors share a canonical structure consisting of an amino terminal domain, a zinc finger DNA binding domain, and a carboxyl terminal ligand binding domain (LBD). The LBD also contains a receptor dimerization interface as well as surfaces that interact with various co-regulatory proteins.

Thyroid hormone response elements (TREs) generally consist of two receptor binding "half sites" related to the sequence AGGTCA arranged as a direct repeat separated by 4 bp, or as an everted repeat or an inverted repeat. By electrophoretic mobility shift assay (EMSA), RXR–TR heterodimers generally are found to bind TREs more strongly than do TR homodimers (Kliewer et al., 1992; Leid et al., 1992; Yu et al., 1991). This difference is especially striking in the presence of thyroid hormone (3,5,3′-triiodothyronine, T3), which destabilizes the TR homodimer–DNA complex (Yen et al., 1992). Thus, it has generally been assumed that RXR–TR heterodimers mediate the regulation of transcription by T3.

However, the importance of RXR for T3 action can be questioned. Using EMSAs, TREs that contain the optimal TR binding half site TAAGGTCA (Katz and Koenig, 1993) can bind TR homodimers well, and the addition of a coactivator protein such as nuclear receptor coactivator 2 (NCoA2/SRC-2/TIF2) stabilizes this TR homodimer-TRE complex, even in the presence of T3 (Diallo et al., 2005). Yeast expression studies suggest that TREs form a spectrum of RXR independence, roughly correlating with the resemblance of the 5′ half site to the optimal TAAGGTCA (Olson and Koenig, 1997). However, it has been difficult to examine the role of RXR in more relevant cellular systems as there are no RXR-null mammalian cells, and it has not been possible to measure TR homodimer versus RXR–TR heterodimer complexes on TREs *in vivo* by chromatin immunoprecipitation.

We therefore employed an RNA interference approach to specifically inhibit RXR activity in a mouse pre-neuronal cell line (Neuro2a) that expresses TRβ1, denoted N2a/TRβ (Denver et al., 1999; Lebel et al., 1994). We then measured the T3 induction of endogenous genes in the presence and absence of RXR

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activity, with the goal of isolating a pool of genes at either end of a spectrum of RXR responsiveness. We show herein that siRNAs against RXRs specifically inhibit RXR activity in this murine cell line, and that there is indeed a hierarchy of RXR requirement among endogenous T3 responsive genes in this system.

## 2. Materials and methods

### 2.1. Cell culture

The mouse pre-neuronal cell line N2a/TRβ has been described (Denver et al., 1999; Lebel et al., 1994). The cells were grown in F-12 Ham's media supplemented with 10% charcoal stripped fetal bovine serum with penicillin–streptomycin and 0.5 mg/ml hygromycin B (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere with 5% $CO_2$.

### 2.2. Transient transfections

Two annealed double stranded siRNAs directed against mouse RXRα and two directed against mouse RXRβ were purchased from Ambion (Austin, TX) as follows: RXRα #1 sense sequence GGAUAUCAAGCCGCCACUAtt, antisense sequence UAGUGGCGGCUUGAUAUCCtc; RXRα #2 sense sequence GGAAAUAUGGCCUCCUUCAtt, antisense sequence UGAAGGAGGCCAUAUUUCCtg; RXRβ #1 sense sequence GGGUUUCUUCAAGCGCACCtt, antisense sequence GGUGCGCUUGAAGAAACCCtt; RXRβ #2 sense sequence GGACCUGACCUACUCGUGUtt, antisense sequence ACACGAGUAGGUCAGGUCCtt. A standard control siRNA which does not recognize any known mammalian gene sequence also was purchased from Ambion (catalog #4611).

Annealed siRNAs were transfected into N2a/TRβ cells in 6-well clusters using siPORT Amine according to the manufacturer's instructions (Ambion). Briefly, siRNAs were diluted into serum free media, then siPORT Amine was added at a ratio of 6 μl to 1 μl of each siRNA from a 50 μM stock. Final siRNA concentrations were 200 nM total for all transfections. N2a/TRβ cells were washed and then incubated with the nucleic acid/siPORT Amine mixture for 3 h. At the end of the incubation, growth media containing 20% stripped fetal bovine serum and antibiotics were added to the transfection media at a 1:1 ratio to result in standard final concentrations. The cells were harvested and assayed within 72 h of transfection.

For experiments in which siRNAs were cotransfected with plasmid DNA, Lipofectamine 2000 was utilized according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Plasmids utilized for transfection experiments include a reporter construct consisting a 3× repeat of the TRE from the rat alpha myosin heavy chain gene (Izumo and Mahdavi, 1988) driving firefly luciferase expression (MHC-Luc), a reporter plasmid consisting of the glucocorticoid responsive MMTV promoter driving firefly luciferase expression (GRE-Luc) (Nordeen, 1988), a transfection control vector expressing *Renilla* luciferase under the control of the CMV promoter (CMV-*Renilla*) (Promega, Madison, WI), an expression vector for murine RXRγ (pCDM-RXRγ), and empty pCDM. Combinations of these plasmids were transfected as noted in the descriptions of individual experiments. siRNAs and plasmids were mixed in serum-free media to which Lipofectamine 2000 was added at a ratio of 2 μl Lipofectamine 2000 to 200 ng plasmid DNA and 200 nM final concentration siRNAs for each well of a 24-well cluster. N2a/TRβ cells were incubated with the nucleic acid/Lipofectamine 2000 mixture for three hours, then cultured in growth media, and harvested after 48 h for analysis. Firefly and *Renilla* luciferase activities were determined using the Dual Luciferase system (cat. #E1960, Promega). Results are expressed as the ratio of firefly luciferase activity normalized to *Renilla* luciferase activity, and are pooled from at least three independent experiments as described above.

### 2.3. RNA processing and Northern blotting

RNA was prepared from cell cultures by extraction with Trizol (Invitrogen) followed by passage over an RNeasy column (Qiagen, Valencia, CA) according to the manufacturer's protocols. Northern blotting was performed using standard protocols with probe labeling using a Strip-EZ DNA Kit (Ambion). Detection was with a BioRad phosphorimager.

### 2.4. Western blot analysis

For Western blotting, cells were washed twice in phosphate buffered saline, then trypsinized, centrifuged, and nuclear proteins extracted using NE-PER (Pierce) according to the manufacturer's protocol. The resulting nuclear proteins were separated by SDS-PAGE in 10% polyacrylamide gels. The proteins were transferred to nitrocellulose membranes and probed using RXRβ antibody #06-527 (Upstate Biotechnology, Charlottesville, VA) at 1:2000 followed by horseradish peroxidase conjugated goat anti-rabbit IgG #sc-2301 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100,000. Detection was with Super Signal West Dura enhanced chemiluminescence detection reagents (Pierce, Rockford, IL) and a BioRad Fluor-S Max Multiimager.

### 2.5. Microarray

RNA was processed in the University of Michigan Cancer Center Affymetrix Core Facility (http://www.michiganmicroarray.com/) using Mouse Genome 430 2.0 GeneChips following the manufacturer's standard protocol. Affymetrix GeneChip data were analyzed as follows. Raw probe-level data were converted to expression measures using the Robust Multi-array Average method, which is implemented in the Affymetrix package of Bioconductor. Briefly, the raw perfect match probes were quantile normalized to remove any non-biological variability (due to differential binding, chip effects, etc.). The normalized probe data were then converted to an expression measure for each gene on each chip using a robust modeling strategy. Differentially expressed genes were detected by fitting a by-gene linear model to the expression values and extracting the contrasts of interest. Standard error estimates were adjusted using an empirical Bayes method to account for small sample size. Contrasts of interest included the comparison of T3 treated and untreated cells with and without siRNA. We also tested for an interaction between these treatments, which tested for genes that reacted differently to the T3 treatment when also treated with siRNA. Genes were selected based on an adjusted $p$-value of 0.005, adjusting for multiplicity using false discovery rate.

### 2.6. Real time PCR

Total RNA prepared as described above was reverse transcribed using random hexamer primers and SuperScript III (Invitrogen). Real time PCR was performed on an Applied Biosystems 7500 thermal cycler using SYBR Green PCR Master Mix according to the vendor's instructions. Cycling conditions were 95 °C for 10 min, then 45 cycles of 94 °C for 15 s, 56 °C for 30 s, and 72 °C for 1 min. The primers used for real time PCR are given in Table 1. For each gene on each reaction plate, a standard curve was generated in duplicate using the cDNA from 1, 10 and 100 ng of RNA, and this was used by the instrument's software to convert cycle threshold values to a quantity corresponding to ng equivalents of starting RNA for each sample analyzed (also in duplicate). In addition to running reactions for each of the T3 inducible target genes, three neutral control genes were studied: glyceraldehyde 3-phosphate dehydrogenase, beta actin and selenoprotein M. The expression of these three genes was independent of culture conditions (siRNA or T3). The expression levels of the three were averaged, and this was used to normalize the expression of each T3 target gene under each culture condition. Separately, real time RT/PCR was used to test whether the RXRα and β siRNAs blocked the expression of RXRγ, which was transfected in reporter gene experiments described in Section 2.2 to rescue the RXR knock down. The primers used to quantify RXRγ were (top strand) TGCTCTTGGCTCTCCGTATAG and (bottom strand) AGCTGCTGACACTGTTGACC.

### 2.7. Promoter luciferase plasmids

The polymerase chain reaction and AccuPrime Pfx polymerase (Invitrogen) were used to amplify promoter fragments from eight genes found to be T3 responsive in the microarray studies. The genes and amplified regions are: *Alas*1 −2058 to +17 (+1 is the start of transcription), *Arvcf*1 −2425 to +28, *Jup* −2930