RXR acts as a coregulator in the regulation of genes of the hypothalamo–pituitary axis by thyroid hormone receptors

L Laflamme, G Hamann, N Messier, S Maltais and M-F Langlois

Department of Physiology and Medicine, Division of Endocrinology, Faculty of Medicine, University of Sherbrooke, CHUS, 3001, 12th Avenue North, Sherbrooke, Quebec, Canada J1H 5N4

(Requests for offprints should be addressed to M-F Langlois; Email: Marie-France.Langlois@Usherbrooke.ca)

Abstract

Thyroid hormone receptors (TRs) often modulate transcriptional activity of target genes by heterodimerization with the 9-cis retinoic acid receptor (RXR). On positive thyroid response elements (TREs), RXR favors binding of the TR–RXR complex to DNA and stimulates transcription. RXR action on negative TREs is unclear. Furthermore, the single half-site configuration of many negative TREs does not favor the binding of a classic TR–RXR heterodimer. In a comparative study using CV-1 cells (relatively RXR- and TR-deficient) and JEG-3 cells (relatively TR-deficient), we demonstrate the importance of RXR in the negative transcriptional regulation of genes of the hypothalamo–pituitary axis by tri-iodothyronine. While RXR has variable effects on ligand-independent activation produced by TRs, it was required for efficient ligand-dependent repression of the TRH gene for TRα1 and TRβ1 and of the TSH genes by all TRs. Using different RXR constructs we also observed the importance of the C-terminus of RXR but not of the N-terminus nor the DNA-binding domain, in the potentiation of negative regulation. We thus suggest that, with regard to negative regulation of the TRH and TSH genes by thyroid hormones, RXR behaves more like a cofactor than a classic heterodimerization partner.

Journal of Molecular Endocrinology (2002) 29, 61–72

Introduction

Thyroid hormones have very important metabolic and developmental effects in nearly all organ systems of the body. They exert most of their action at the genomic level, through binding to thyroid hormone receptors (TRs), which are well conserved transcription factors of the nuclear receptor superfamily (see Yen 2001 for review). The transcription of many genes is regulated by TRs, a good number of which have been studied in detail. Most of our current knowledge is based on the study of positively regulated genes, such as those for growth hormone (Glass et al. 1987), malic enzyme (Desvergne et al. 1991), Na+/K+-ATPase (Gick & Ismail-Beigi 1990), sarcoplasmic endoplasmic reticulum Ca2+-ATPase (Hartong et al. 1994), and many others. In contrast, fewer genes are negatively regulated by tri-iodothyronine (T3), like those of the hypothalamo–pituitary axis: thyrotropin-releasing hormone (TRH) (Hollenberg et al. 1995), thyrotropin (TSH) α (glycoprotein hormone common subunit) (Burnside et al. 1989) and TSHβ (Bodenner et al. 1991, Carr & Wong 1994). The limited studies on these genes have demonstrated distinct features of negative regulation by thyroid hormone, such as ligand-independent activation (LIA) and T3-dependent repression (LDR) (Wondisford et al. 1989, Carr & Wong 1994, Hollenberg et al. 1995). Moreover, negative regulation plays a pivotal role in thyroid hormone action as both TRH and TSH set the level of activity of the thyroid gland.

In vertebrates, TR isoforms derive from two genes: c-erbAα and c-erbAβ (Yen 2001). By alternative RNA splicing, c-erbAα gives rise to TRα1 and the c-erbAα2 protein, which does not bind T3 (Koenig et al. 1989, Katz & Koenig 1994,
Liu et al. (1995). Using alternative 5′ exons and separate promoters, TRβ1 and TRβ2 are derived from the c-erbAβ locus (Hodin et al. 1989, Wood et al. 1994). TRβ isoforms differ structurally only in their amino-terminal domain, which derive from distinct exons and are completely different. TRβ2 expression is restricted to the pituitary (Hodin et al. 1989) and hypothalamus (Lechan et al. 1994), although low mRNA and protein levels have been found in other parts of the central nervous system and some peripheral tissues (Lechan et al. 1993, Schwartz et al. 1994, Li & Boyages 1996). This is in contrast to other TR isoforms which have ubiquitous expression (Sakurai et al. 1989, Schwartz et al. 1992). It has previously been shown that TRβ2 plays a unique role in negative regulation by thyroid hormone. It is the only TR isoform to achieve significant LIA of the TRH and TSHα genes and LDR of the TRH gene (Langlois et al. 1997). The TRβ2 knockout mice confirm the importance of this isoform for in vivo regulation of the hypothalamo–pituitary axis (Abel et al. 2001).

The 9-cis retinoic acid receptor (RXR), another member of the nuclear receptor superfamily, is a major heterodimeric partner for many nuclear receptors, including TRs, and serves as a modulator of their transcriptional regulation (see Mangelsdorf & Evans 1995 for review). There are three human RXR isoforms: RXRa, RXRβ and RXRγ (Hamada et al. 1989, Mangelsdorf et al. 1990, Leid et al. 1992, Mangelsdorf et al. 1992). RXRγ is of particular interest in the study of negative regulation since, in contrast to mRNA of the other isoforms, which are widely expressed, RXRγ expression is restricted to a few tissues and is predominant in TSH-producing cells of the pituitary gland (Sugawara et al. 1995, Haugen et al. 1997, Sanno et al. 1997). RXRa is also expressed at high levels in the human pituitary (Sanno et al. 1997), where RXRβ was not found, although it is expressed in rat pituitary tissue (Sugawara et al. 1995, Sanno et al. 1997).

The role of RXR in the context of positively regulated genes has been extensively studied. RXR enhances stimulatory T3 responses from positive thyroid response elements (pTREs) and facilitates DNA binding (Rosen et al. 1992, Zhang et al. 1992, Leng et al. 1994, Hsu et al. 1995, Nelson et al. 1996). However, the effect of RXR on negative thyroid response elements (nTREs) has not been the subject of thorough investigation. Haugen et al. (1997) have shown that RXRγ is necessary to suppress TSHβ promoter activity in TR-T97 thyrotropic tumor cells, suggesting an important role in negative regulation. In this study, we demonstrate the importance of RXR in the regulation of the genes of the hypothalamo–pituitary axis by thyroid hormone. Using different RXR constructs we also demonstrate that the function of RXR in negative regulation is located in its C-terminus and does not involve its binding to DNA. We thus suggest that RXR acts more like a cofactor than a classic heterodimerization partner, in the context of negative regulation by thyroid hormone. These results emphasize the importance of RXR, not only on positively regulated genes but also on negative regulation by thyroid hormones.

Materials and methods

Plasmid construction

The cDNAs of human (h) TRα1, hTRβ1, hTRβ2 (Fig. 1A), hRXRα, hRXRβ, hRXRγ (Fig. 4A) and hRAR (Fig. 6A) were inserted into pSG5 (Stratagene, La Jolla, CA, USA), which is the expression vector used for all receptor constructs utilized in this report (Breathnach & Harris 1983). The RXR-ΔC mutant (Fig. 6A) was created by deleting the amino acids 402–462 of hRXRα with the StuI restriction enzyme. RXR E453A (Fig. 6A) was produced by using an in vitro mutagenesis kit (Promega) and a mutagenesis primer that changed the glutamic acid to alanine at codon 457 in the AF-2 region. The RAR-RXR construct (Fig. 6A) was generated from hRAR for which the C-terminus had been swapped for hRXRα C-terminus. GAL4 and GAL4-RXR encode amino acids 1–147 of GAL4 DNA-binding sequence alone or fused to the C-terminus of hRXRα (amino acids 188–462), in the pBXG1 vector which contains the GAL4 nuclear localization signal; for cloning purposes an EcoR1 site has been introduced in frame, by PCR, just 3′ to the DNA-binding domain of RXRα, and the PCR product is introduced in frame in the EcoR1 site of the vector. Mutating the first zinc finger of the DNA-binding domain of TRβ1 for the corresponding glucocorticoid receptor sequence created the TRβ1-DNA binding mutant (TRβ1-DBM).

The nTRE reporter constructs included the 5′-flanking sequences from the human TRH
(−900/+55) (Hollenberg et al. 1995), the common glycoprotein α-subunit (TSHα, −846/+26) and the TSH β-subunit (TSHβ, −125/+37) (Chatterjee et al. 1989, Bodenner et al. 1991) fused upstream of a luciferase reporter gene (PSVO or PGL3 vector). The RARE3 reporter contains 3 copies of an idealized retinoic acid response element (RARE) fused to luciferase in the PSVO vector (Kurlandsky et al. 1994).

**Cell culture and transfection assays**

The CV-1 (African green monkey renal carcinoma, ATCC, Manassas, VA, USA) and JEG-3 (human choriocarcinoma, ATCC) cell lines were maintained in DMEM supplemented with 10% FBS, penicillin, streptomycin, and amphotericin (Gibco BRL). Transient transfections were realized in 6-well plates on subconfluent cells using the calcium-phosphate technique. Each experiment utilizes 1·6 µg reporter construct and 80 ng of each receptor expression vector or vector alone per well. Sixteen hours after transfection, culture medium is replaced by medium containing FBS stripped of hormones by anion-exchange resin and charcoal, and with or without 10 nM T3. Thirty-six to forty hours after transfection, cells are harvested and assayed for luciferase activity. Data are from at least three independent experiments performed in triplicate, and are displayed as means ± s.e.

**RNA isolation and analysis**

Total RNA was isolated either from CV-1 or JEG-3 cells using the TRIzol Reagent (Sigma). Ten micrograms glyoxylated RNA were electrophoresed on a 1% agarose gel and analyzed by Northern blot hybridization. The blot was hybridized with a \(^{32}\)P-random labeled cDNA probe for a common region of the different isoforms of hRXR generated using a Nde1-Pst1 fragment of hRXRα. The blot was stripped and hybridized with a probe for human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH), for loading control.

**Immunoprecipitation and immunoblotting**

CV-1 cells grown in 100-mm Petri dishes were transfected with 500 ng hTRβ1 and hRXRα. Cells were lysed in 10 mM Tris, 10 mM NaCl, 3 mM MgCl\(_2\), 0·5% NP40 and centrifuged at 500 × g for 10 min. The pellet was resuspended in nuclear extract buffer (15 mM Tris, 60 mM KCl, 15 mM NaCl, 1 mM EDTA, 0·2 mM EGTA, 1 mM CaCl\(_2\), 10 mM MgCl\(_2\), 0·5% SDS). Immunoprecipitation was performed using a polyclonal antibody to the full length thyroid hormone receptor of chicken origin (anti-TRα1 FL-408: sc-772; Santa Cruz Biotechnology, Santa Cruz, CA, USA), recognizing TRα1 and TRβ1 of mouse, rat human and chicken origin. After overnight incubation of sample at 4 °C with rocking, protein G-sepharose was added, and samples were rocked for 2 h at 4 °C. After five washes with nuclear extract buffer without detergent, proteins were resolved on a 10% SDS-polyacrylamide gel and transferred to a polyvinylene difluoride membrane. The membrane was blocked with 1% gelatin, 0·05% Tween 20 in TBS buffer, pH 7·5 and incubated overnight at 4 °C with constant agitation with a monoclonal anti-TRα1 (C4) antibody (sc-740; Santa Cruz Biotechnology). This antibody reacts with both TRα1 and TRβ1 of human origin. The membrane was washed with TBS/Tween 20. Detection was accomplished using horse-radish peroxidase-conjugated anti-mouse antibody (Amersham, Canada) and enhanced chemiluminescence detection system (Roche Molecular Biochemicals, Canada).

RXR protein expression was analyzed using 75 µg of nuclear extracts of CV-1 or JEG-3 cells. Proteins were electrophoresed on a 12% SDS-polyacrylamide gel and immunoblotted using an anti-RXRα antibody (sc-774; Santa Cruz Biotechnology), recognizing all RXR isoforms: RXRα, RXRβ and RXRγ. The different RXR isoforms used as controls were made by in vitro translation in rabbit reticulocyte lysate (TNT kit, Promega) using RXRα, RXRβ or RXRγ cDNAs in pSG5 and T7 polymerase.

**Results**

**RXR is a cell-specific factor enhancing negative regulation**

To investigate the role of RXR in negative regulation by thyroid hormone, we first studied the regulation of nTREs in two cell lines: CV-1 and JEG-3. The effect of TRs on negatively regulated genes has two components: LIA and LDR. Since it was previously shown that TR isoforms have variable actions on nTREs (Langlois et al. 1997,
Safer et al. (1997), the three isoforms were studied separately and are schematically represented in Fig. 1A.

In CV-1 cells, TRβ2 produced a higher LIA than TRα1 and TRβ1 on the TRH reporter (Fig. 1B). TRβ2 was also the only isoform to produce significant LDR (Fig. 1B and C). Cotransfection of RXRα in these cells was beneficial for LIA of TRα1 and TRβ1 receptors and increased the negative regulation of the TRH reporter by all three TR isoforms (Fig. 1B and C). Interestingly, repression of the TRH reporter under transcriptional levels reached with the pSG5 vector alone was only achieved in the presence of RXRα (Fig. 1B). Overexpression of RXRα in CV-1 cells had no influence on the TR expression levels compared with cells transfected only with TRβ1, as shown by Western blot experiment (Fig. 2A).

Transcriptional regulation by TRs was different in JEG-3 cells as compared with CV-1 cells. Individual LIA produced by TRα1 and TRβ1 were similar in both cell lines, while TRβ2 failed to produce any significant LIA in JEG-3 cells (Fig. 1D). All three isoforms were able to repress transcription of the TRH reporter in the presence of T3 (Fig. 1D and E). The fold repression observed in RXRα-transfected CV-1 cells reached comparable levels to those obtained with JEG-3 cells, except for the TRα1 receptor (Fig. 1C and E).

Evaluation of the RXR content of JEG-3 and CV-1 cells by Northern and Western blot experiments revealed a high endogenous expression of RXRα in JEG-3 cells, whereas CV-1 cells contained no detectable RXR mRNA or protein (Fig. 2B and C). This is consistent with a previous report showing that RXRα is the major isoform expressed in JEG-3 cells (Guibourdenche et al. 1998).

The overexpression of RXRα in JEG-3 cells abolished the LIA of all three TR isoforms and, in contrast to CV-1 cells, repression in the presence of T3 was not increased by cotransfection of RXRα (Fig. 1D and E). Lowering of the transcriptional activities seen in JEG-3 cells overexpressing RXRα suggest a squelching effect of the transcriptional machinery.

On the TSHα reporter, TRβ2 is the only isoform to produce significant LIA in the absence of RXR, reaching 1.38-fold activation compared with 1.12- and 1.14-fold for TRα1 and TRβ1 (Fig. 3A). RXRα cotransfection is needed to increase LIA and LDR of all TR isoforms (Fig. 3A and B). Similar results are obtained for the TSHβ reporter (Fig. 3C and D), suggesting that RXR is important for the regulation of all nTREs. In JEG-3 cells, TSHα and TSHβ reporters are negatively regulated by all TR isoforms and transcription is not significantly modified by cotransfection of RXR (data not shown). Altogether, these results demonstrate the importance of RXR in negative regulation of genes of the hypothalamo–pituitary axis, especially by TRα1 and TRβ1.

**Potency of RXR isoforms**

In Fig. 4A a schematic representation of the different RXR isoforms is shown. On a retinoic acid response element (RARE), all RXR isoforms produce similar activation of transcription in the presence of 9-cis retinoic acid (Fig. 4B). There is also transactivation with the pSG5 vector alone, suggesting the presence of endogenous RAR in CV-1 cells.

All three RXR isoforms greatly increase the efficiency of T3-mediated repression of the TRH gene (Fig. 5A). Furthermore, because TSHα and TSHβ genes are expressed in the pituitary in vivo and could be regulated differently from TRH (expressed in the hypothalamus), especially by RXRγ, we found it important to compare the function of RXR isoforms on these genes. On TSHα and TSHβ nTREs, all RXR isoforms enhance negative regulation, but RXRβ is slightly weaker in this respect (Fig. 5B and C).

**The C-terminus of RXR is necessary for its action**

It is known from previous work that RXR heterodimerizes with TR through its C-terminal EF domain and the importance of this interaction was evaluated as regard to positive regulation (Leng et al. 1994). We aimed to identify the areas of RXR that are necessary for negative regulation, and to do so we designed a series of RXR mutants that are represented in Fig. 6A. Transfection data using these RXR mutants are shown in Fig. 6B. We determined that a carboxy-terminal deletion mutant of RXR lacking the heterodimerization domain (RXR-ΔC) lost its ability to enhance repression of the TRH reporter. However, a mutation of the AF-2 domain of RXR (E453A)
RXR is a cell-specific factor enhancing negative regulation. (A) Schematic representation of the human thyroid hormone receptor (TR) isoforms used in these studies. (B) and (C) Effect of TR isoforms and RXRα on transcriptional regulation of the TRH reporter, in the absence and presence of T3, in CV-1 cells. (D) and (E) Effect of TR isoforms and RXRα on transcriptional regulation of the TRH reporter, in the absence and presence of T3, in JEG-3 cells. The data are expressed in fold repression or in relative activity, compared with the empty pSG5 vector in the absence of T3. ±S.E.M. An asterisk indicates that the difference between the pairs denoted is significant at a confidence level of $P \leq 0.05$ by Student’s t-test. A # indicates that the difference from the control is significant at a confidence level of $P \leq 0.05$ by Student’s t-test.

Figure 1
does not affect its function. RAR is not a classic heterodimeric partner of TRs and, by itself, produced no effect on negative regulation in our system. In contrast, the RAR–RXR chimera in which the EF domain of RXR is fused to the A-D domains of RAR shows a similar increase of repression by T₃ as compared with RXR/afii9825. These data indicate that the heterodimerization domain of RXR is required to enhance negative regulation by T₃ and further suggests the notion that the DNA-binding domain of RXR is not necessary to mediate its action.

Negative regulation by thyroid hormone requires TR, but not RXR DNA-binding

To confirm that RXR could enhance negative regulation in the absence of its DNA-binding and amino-terminal domains, we designed an RXR construct in which the C-terminus of RXR was fused to a heterologous GAL4 DNA-binding domain in an expression vector that ensures nuclear localization. This RXRα-GAL construct produces repression comparable to that of RXRα wild type (wt) on the TRH reporter and also on TSHα and TSHβ reporters (Fig. 7A). The GAL4 DNA-binding domain being unable to recognize TREs, our results suggest that the C-terminus of RXR is sufficient to mediate negative regulation and that it functions without forming a classic heterodimer on the nTRE.

Since the DNA-binding domain of RXR was facultative, we then generated a TRβ1 construct without DNA-binding properties (TRβ1-DBM) to investigate the possibility of the effect occurring in solution. The results presented in Fig. 7B clearly show that TRβ1 binding to the TRH nTRE is essential for negative regulation of this gene, as the TRβ1-DBM does not induce repression either in the presence or absence of RXRα. Similar results are obtained with the TSHα and TSHβ genes (data not shown).
Discussion

The importance of RXR in the regulation of positive thyroid hormone response elements has been well demonstrated. On pTREs, RXR binds a half site of the TRE and increases TR DNA-binding and transactivation potency (Rosen et al. 1992, Zhang et al. 1992, Leng et al. 1994, Hsu et al. 1995, Mangelsdorf & Evans 1995, Nelson et al. 1996). However, the role of RXR in the regulation of nTREs has been less studied. The configuration of many nTREs does not favor the binding of a classic TR-RXR heterodimer since they often bear a single consensus half site, which implies that only the TR would be able to bind DNA as a monomer. In this report we indicate the importance of RXR in the negative regulation of genes of the hypothalamo–pituitary axis. We also propose a
novel model of interaction between TRs and RXR on negative thyroid hormone response elements naturally found in the TRH and TSH genes.

The results we obtained on negatively regulated promoters show that RXRα enhances the LIA of the TSH genes by all three TR isoforms. On the TRH gene, RXRα is beneficial for the LIA produced only by the TRα1 and TRβ1 isoforms, suggesting a distinct mode of regulation of this promoter. Furthermore, as opposed to the TSH genes, absolute repression of the TRH gene, under levels reached with the pSG5 vector alone was obtained only when RXRα was expressed. For all the negatively regulated genes of the hypothalamo–pituitary axis studied, RXRα accentuates fold repression by every TR, therefore acting as an

**Figure 4** Comparison of RXR isoforms on a retinoic acid response element (RARE). (A) Schematic representation of 9-cis retinoic acid receptor (RXR) isoforms. (B) Effect of empty vector pSG5 and RXR isoforms on an idealized RARE reporter, in the absence and presence of 9-cis retinoic acid (9-cis RA). The data are expressed in relative activity, compared with pSG5 empty vector without T3, ± S.E.M. An asterisk indicates that the difference between the pairs is significant with a confidence level of $P \leq 0.001$ by Student’s t-test. A # indicates that the difference from the control is significant at a confidence level of $P \leq 0.05$ by Student’s t-test.

**Figure 5** Comparison of RXR isoforms in negative regulation by thyroid hormone. Fold T3-induced repression by TR isoforms in conjunction with RXR isoforms on (A) the TRH reporter, (B) the TSHα reporter and (C) the TSHβ reporter, in CV-1 cells. The data are expressed in fold repression ± S.E.M. An asterisk indicates that the difference from the control is significant at a confidence level of $P \leq 0.05$ by Student’s t-test.
important transcriptional regulator. In the absence of RXR, TRβ2 behaves differently from TRα1 or TRβ1. On the TRH gene, TRβ2 was the only isoform to show significant LDR whereas on the TSH genes, it was unique in showing LIA. Different behavior of this receptor on negative regulation may derive from its distinct amino-terminus, interacting either differently with known coregulators or with new cofactors (Langlois et al. 1997, Yang et al. 1999).

While we have shown that RXR is an important cell-specific factor for the regulation of TRH, TSHα and TSHβ genes, two other groups have come to different conclusions based on studies realized in other experimental conditions. Indeed, Hallenbeck et al. (1993) and Carr and Wong (1994) have reported that RXR inhibits negative regulation by T3. However, they have not excluded an artifact in their CAT assay. Also, another group has shown that RXR had no effect on negative regulation of the TSHα promoter by TRβ1 wt (Takeda et al. 1997). However, experiments were conducted in JEG-3 cells already expressing RXR at high level, which may have caused squelching of the effects, as we observed in our own experiments with JEG-3 cells (Fig. 1D and E). Our results are in agreement with those of Haugen et al. (1997) who have shown the importance of RXRγ in

![Figure 6](image)

**Figure 6** The C-terminus of RXR is necessary to mediate its action. (A) Schematic representation of RXR mutants utilized in these studies. (B) Effect of RXR and RAR wt and constructs on fold T3-induced repression of the TRH reporter, in CV-1 cells. The data are expressed in fold repression ± S.E.M. An asterisk indicates the difference from the control is significant with a confidence level of $P \leq 0.001$ by Student’s t-test.

![Figure 7](image)

**Figure 7** Negative regulation by T3 requires DNA binding of TR but the C-terminus of RXR is sufficient to mediate its action. (A) Effect of RXRα and GAL-RXR on T3-induced repression (fold) by TRβ1 of the TRH reporter in CV-1 cells. The data are expressed in relative activity, compared with GAL empty vector without T3, ± S.E.M. (B) Effect of TRβ1 wt and TR DNA-binding mutant (DBM), in the absence and presence of RXR on fold T3-induced repression of the TRH reporter. The data are expressed in fold repression ± S.E.M. An asterisk indicates the difference from the control is significant with a confidence level of $P \leq 0.05$ by Student’s t-test.
suppressing TSHβ promoter activity and thus strongly suggest an important role for RXR in the regulation of the hypothalamo–pituitary axis.

Since RXR isoforms are expressed from three different genes, denoted α, β and γ, we have also examined the role of each different human RXR isoform. RXRα and RXRγ were found to be equally potent in enhancing negative regulation while RXRβ was less efficient. This question has less physiological relevance since RXRβ was not shown to be expressed in human pituitary tissues (Sanno et al. 1997).

Our results strongly suggest a very novel mechanism of action for RXR in which the carboxy-terminal end is essential and sufficient for its action (Figs 6 and 7A). This implies that the RXR portion of the heterodimer does not need to bind the response element and acts more as a coregulator. To our knowledge, it is the first time that such a mechanism of action for RXR has been suggested, and this is consistent with the known configuration of nTREs. In contrast to pTREs, which are always composed of two half-sites, most nTREs described do not seem to have repeated motifs (Burnside et al. 1989, Bodenner et al. 1991, Carr & Wong 1994, Hollenberg et al. 1995, Yen 2001), making it improbable that classic heterodimers could bind these response elements.

The importance of coregulators has been well described for pTREs, while both the involvement and mechanisms of action are still poorly defined for nTREs (Yen 2001). Some studies have suggested the implication of the NcoR corepressor and histone acetylation in negative regulation of the TSHα promoter by TRs (Tagami et al. 1997, 1999). Although the mechanism is not clearly defined, it does not seem to involve a direct interaction of the receptors with DNA. On the other hand, NcoRI, which is devoid of repression domains, has been shown to enhance LIA of a TRH reporter by TRα1 and TRβ1 receptor (Hollenberg et al. 1996). Conformational changes of RXR upon heterodimerization with the TR have been shown to modify the conformation of helix 12 of the RXR and to unmask a corepressor-binding site (Zhang et al. 1999). The absence of DNA-binding of RXR, observed in our study, is not expected to have profound effects on the conformation of both receptors although it may modify their interactions with coactivators and corepressors. However, the fact that the effect of RXR on negative regulation is preserved with the use of the RXR AF-2 mutant (RXR E453A), a mutant previously shown not to bind coactivators, suggests that the effect observed is independent of coactivator binding (McKenna et al. 1999). To clarify the molecular mechanism of negative regulation of genes by TRs, the interactions between coregulators and the TR-RXR complex should be studied further.

One other potential mechanism that could explain the RXR’s role in negative regulation may be by direct interaction with the TATA-binding protein via its AF-2 region, thereby mediating hormone-dependent transactivation, as previously described (Schulman et al. 1995). To verify this, we used the AF-2 mutant of RXR, E453A, which has previously been shown to lack AF-2 function (Liu et al. 1998). This mutant and RXRα had similar activity in our system, suggesting that the AF-2 domain of RXR is not implicated in negative regulation by thyroid hormone.

Finally, we have demonstrated that thyroid hormone receptor DNA-binding is required for transcriptional repression of genes of the hypothalamo–pituitary axis (Fig. 7B). There is still controversy regarding the mechanism underlying negative regulation by thyroid hormone. One hypothesis suggests that negative regulation takes place in solution by squelching of transcription factors (Tagami et al. 1997). Another hypothesis, supported by this report, implicates DNA binding of thyroid hormone receptor on their differently configured negative response elements.

In conclusion, the data presented here confirm the importance of RXR in the negative regulation of the TRH and TSH genes by thyroid hormone. The data also delineate a new model of interaction between TR and RXR on these negatively regulated promoters. In this new model, TR interacts with the C-terminus of RXR, without any binding of the RXR moiety to DNA. Therefore, on these negatively regulated genes, RXR behaves as a coregulator instead of a classic heterodimerization partner.

Acknowledgements

This work has been supported in part by grants and scholarships from the Banting Foundation, Medical Research Council of Canada (15655) and Fonds de
Recherche en Santé du Québec to M-F L and by a scholarship from the Thyroid Foundation of Canada to L L. This work was presented in part at the 70th Annual Meeting of the American Thyroid Association, 2000 CDA/CSEM Annual Meeting and the 11th International Congress of Endocrinology. We would like to thank Dr Fredric E Wondisford for plasmids, fruitful discussions and the critical review of this manuscript. We would also like to thank Dr P Chambon for cDNAs of RXRβ and RXRγ.

References


Breathnach R & Harris BA 1983 Plasmids for the cloning and expression of full-length double-stranded cDNAs under control of the SV40 early or late gene promoter. *Nucleic Acids Research* 11 7119–7136.


Langlois MF, Zanger K, Monden T, Safer JD, Hollenberg AN & Wondisford FE 1997 A unique role of the β2 thyroid hormone receptor isoform in negative regulation by thyroid hormone - mapping of a novel amino-terminal domain important for ligand-independent activation. *Journal of Biological Chemistry* 272 24927–24933.


Lechan RM, Qi Y, Jackson IMD & Mahdavi V 1994 Identification of thyroid hormone receptor isoforms in thyrotropin-releasing hormone neurons of the hypothalamic paraventricular nucleus. *Endocrinology* 135 92–100.


Lechan RM, Qi Y, Jackson IMD & Mahdavi V 1994 Identification of thyroid hormone receptor isoforms in thyrotropin-releasing hormone neurons of the hypothalamic paraventricular nucleus. *Endocrinology* 135 92–100.

Liu RT, Suzuki S, Miyamoto T, Takeda T, Ozata M & D’Groot IJ 1995 The dominant negative effect of thyroid hormone receptor...
splicing variant α2 does not require binding to a thyroid response element. *Molecular Endocrinology* 9 86–95.


Nelson CC, Hendy SC, Faris JS & Romaniuk PJ 1996 Retinoid X receptor alters the determination of DNA-binding specificity by the P-box amino acids of the thyroid hormone receptor. *Journal of Biological Chemistry* 271 19464–19474.


Tagami T, Madison LD, Nagaya T & Jameson JL 1997 Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone. *Molecular and Cellular Biology* 17 2642–2648.

Tagami T, Park Y & Jameson JL 1999 Mechanisms that mediate negative regulation of the thyroid-stimulating hormone α gene by thyroid hormone receptor. *Journal of Biological Chemistry* 274 22345–22353.


Wood WM, Dowling JM, Haugen BR, Bright TM, Gordon DF & Ridgway EC 1994 Structural and functional characterization of the genomic locus encoding the murine β2 thyroid hormone receptor. *Molecular Endocrinology* 8 1605–1617.


Yen PM 2001 Physiological and molecular basis of thyroid hormone action. *Physiological Reviews* 81 1097–1142.


Received in final form 11 April 2002
Accepted 25 April 2002