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

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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 TR α 1 and TR β 1 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.

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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 TSH β (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-erbA α and c-erbA β (Yen 2001). By alternative RNA splicing, c-erbA α gives rise to TR α 1 and the c-erbA α 2 protein, which does not bind T₃ (Koenig *et al.* 1989, Katz & Koenig 1994,

Liu *et al.* 1995). Using alternative 5' exons and separate promoters, TR β 1 and TR β 2 are derived from the c-erbA β locus (Hodin *et al.* 1989, Wood *et al.* 1994). TR β isoforms differ structurally only in their amino-terminal domain, which derive from distinct exons and are completely different. TR β 2 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 TR β 2 plays a unique role in negative regulation by thyroid hormone. It is the only TR isoform to achieve significant LIA of the TRH and TSH α genes and LDR of the TRH gene (Langlois *et al.* 1997). The TR β 2 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: RXR α , RXR β and RXR γ (Hamada *et al.* 1989, Mangelsdorf *et al.* 1990, Leid *et al.* 1992, Mangelsdorf *et al.* 1992). RXR γ is of particular interest in the study of negative regulation since, in contrast to mRNA of the other isoforms, which are widely expressed, RXR γ 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). RXR α is also expressed at high levels in the human pituitary (Sanno *et al.* 1997), where RXR β 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 T_3 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 RXR γ is necessary to suppress TSH β promoter activity in TtT-97 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) TR α 1, hTR β 1, hTR β 2 (Fig. 1A), hRXR α , hRXR β , hRXR γ (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- Δ C mutant (Fig. 6A) was created by deleting the amino acids 402–462 of hRXR α with the *Stu*I 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 hRXR α C-terminus. GAL4 and GAL4-RXR encode amino acids 1–147 of GAL4 DNA-binding sequence alone or fused to the C-terminus of hRXR α (amino acids 188–462), in the pBXG1 vector which contains the GAL4 nuclear localization signal; for cloning purposes an *Eco*RI site has been introduced in frame, by PCR, just 3' to the DNA-binding domain of RXR α , and the PCR product is introduced in frame in the *Eco*RI site of the vector. Mutating the first zinc finger of the DNA-binding domain of TR β 1 for the corresponding glucocorticoid receptor sequence created the TR β 1-DNA binding mutant (TR β 1-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 (TSH α , −846/+26) and the TSH β -subunit (TSH β , −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 μ g 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 hormone by anion-exchange resin and charcoal, and with or without 10 nM T_3 . 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 $\pm$ 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 NdeI-PstI fragment of hRXR α . 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 hTR β 1 and hRXR α . Cells were lysed in 10 mM Tris, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP40 and centrifuged at 500 $\times$ 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 CaCl₂, 10 mM MgCl₂, 0.5% SDS). Immunoprecipitation was performed using a polyclonal antibody to the full length thyroid hormone receptor of chicken origin (anti-TR α 1 FL-408: sc-772; Santa Cruz Biotechnology, Santa Cruz, CA, USA), recognizing TR α 1 and TR β 1 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 polyvinylidene 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-TR α 1 (C4) antibody (sc-740; Santa Cruz Biotechnology). This antibody reacts with both TR α 1 and TR β 1 of human origin. The membrane was washed with TBS/Tween 20. Detection was accomplished using horseradish peroxidase-conjugated anti-mouse antibody (Amersham, Canada) and enhanced chemiluminescence detection system (Roche Molecular Biochemicals, Canada).

RXR protein expression was analyzed using 75 μ g of nuclear extracts of CV-1 or JEG-3 cells. Proteins were electrophoresed on a 12% SDS-polyacrylamide gel and immunoblotted using an anti-RXR α antibody (sc-774; Santa Cruz Biotechnology), recognizing all RXR isoforms: RXR α , RXR β and RXR γ . The different RXR isoforms used as controls were made by *in vitro* translation in rabbit reticulocyte lysate (TNT kit, Promega) using RXR α , RXR β or RXR γ 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, TR β 2 produced a higher LIA than TR α 1 and TR β 1 on the TRH reporter (Fig. 1B). TR β 2 was also the only isoform to produce significant LDR (Fig. 1B and C). Cotransfection of RXR α in these cells was beneficial for LIA of TR α 1 and TR β 1 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 RXR α (Fig. 1B). Overexpression of RXR α in CV-1 cells had no influence on the TR expression levels compared with cells transfected only with TR β 1, 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 TR α 1 and TR β 1 were similar in both cell lines, while TR β 2 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 T_3 (Fig. 1D and E). The fold repression observed in RXR α -transfected CV-1 cells reached comparable levels to those obtained with JEG-3 cells, except for the TR α 1 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 RXR α 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 RXR α is the major isoform expressed in JEG-3 cells (Guibourdenche *et al.* 1998).

The overexpression of RXR α in JEG-3 cells abolished the LIA of all three TR isoforms and, in contrast to CV-1 cells, repression in the presence of T_3 was not increased by cotransfection of RXR α (Fig. 1D and E). Lowering of the transcriptional activities seen in JEG-3 cells overexpressing RXR α suggest a squelching effect of the transcriptional machinery.

On the TSH α reporter, TR β 2 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 TR α 1 and TR β 1 (Fig. 3A). RXR α cotransfection is needed to increase

LIA and LDR of all TR isoforms (Fig. 3A and B). Similar results are obtained for the TSH β reporter (Fig. 3C and D), suggesting that RXR is important for the regulation of all nTREs. In JEG-3 cells, TSH α and TSH β 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 TR α 1 and TR β 1.

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 T_3 -mediated repression of the TRH gene (Fig. 5A). Furthermore, because TSH α and TSH β genes are expressed in the pituitary *in vivo* and could be regulated differently from TRH (expressed in the hypothalamus), especially by RXR γ , we found it important to compare the function of RXR isoforms on these genes. On TSH α and TSH β nTREs, all RXR isoforms enhance negative regulation, but RXR β 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- Δ C) lost its ability to enhance repression of the TRH reporter. However, a mutation of the AF-2 domain of RXR (E453A)

Figure 1 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 RXR α on transcriptional regulation of the TRH reporter, in the absence and presence of T_3 , in CV-1 cells. (D) and (E) Effect of TR isoforms and RXR α on transcriptional regulation of the TRH reporter, in the absence and presence of T_3 , 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 T_3 , $\pm$ 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 2 (A) Western blot analysis of immunoprecipitated TR from CV-1 cells. TRs immunoprecipitated (IP) with rabbit TR specific antibody FL408 were immunoblotted (IB) using mouse TR specific antibody C4. (B) Northern blot analysis of RXR expression in CV-1 and JEG-3 cells. The blot was sequentially hybridized with a cDNA probe made from a fragment of RXR γ common to all RXR isoforms, and a hGAPDH cDNA probe. (C) Western blot analysis of RXR from nuclear extracts of CV-1 and JEG-3 cells. RXR α , RXR β and RXR γ proteins made by *in vitro* translation in rabbit reticulocytes were used as controls to confirm the recognition of all RXR isoforms by the antibody.

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 T_3 as compared with RXR α . These data indicate that the heterodimerization domain of RXR is required to enhance negative regulation by T_3 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 RXR α –GAL construct produces repression comparable to that of RXR α wild type (wt) on the TRH reporter and also on TSH α and TSH β 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 TR β 1 construct without DNA-binding properties (TR β 1–DBM) to investigate the possibility of the effect occurring in solution. The results presented in Fig. 7B clearly show that TR β 1 binding to the TRH nTRE is essential for negative regulation of this gene, as the TR β 1–DBM does not induce repression either in the presence or absence of RXR α . Similar results are obtained with the TSH α and TSH β genes (data not shown).

Figure 3 Importance of RXR in thyroid hormone regulation of TSH α and TSH β genes. Effect of TR isoforms and RXR α cotransfected in CV-1 cells on the regulation of (A) and (B) the TSH α reporter, and (C) and (D) the TSH β reporter. The data are expressed in fold repression or relative activity, compared with pSG5 alone without T_3 , $\pm$ 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.

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

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 T_3 , $\pm$ 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.

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 RXR α enhances the LIA of the TSH genes by all three TR isoforms. On the TRH gene, RXR α is beneficial for the LIA produced only by the TR α 1 and TR β 1 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 RXR α was expressed. For all the negatively regulated genes of the hypothalamo-pituitary axis studied, RXR α accentuates fold repression by every TR, therefore acting as an

Figure 5 Comparison of RXR isoforms in negative regulation by thyroid hormone. Fold T_3 -induced repression by TR isoforms in conjunction with RXR isoforms on (A) the TRH reporter, (B) the TSH α reporter and (C) the TSH β reporter, in CV-1 cells. The data are expressed in fold repression $\pm$ 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.

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 T_3 -induced repression of the TRH reporter, in CV-1 cells. The data are expressed in fold repression $\pm$ S.E.M. An asterisk indicates that the difference from the control is significant with a confidence level of $P \leq 0.001$ by Student's t -test.

important transcriptional regulator. In the absence of RXR, TR β 2 behaves differently from TR α 1 or TR β 1. On the TRH gene, TR β 2 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, TSH α and TSH β 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 T_3 . 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 TSH α promoter by TR β 1 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 RXR γ in

Figure 7 Negative regulation by T_3 requires DNA binding of TR but the C-terminus of RXR is sufficient to mediate its action. (A) Effect of RXR α and GAL-RXR on T_3 -induced repression (fold) by TR β 1 of the TRH reporter in CV-1 cells. The data are expressed in relative activity, compared with GAL empty vector without T_3 , $\pm$ S.E.M. (B) Effect of TR β 1 wt and TR DNA-binding mutant (DBM), in the absence and presence of RXR on fold T_3 -induced repression of the TRH reporter. The data are expressed in fold repression $\pm$ S.E.M. An asterisk indicates that the difference from the control is significant with a confidence level of $P \leq 0.05$ by Student's t -test.

suppressing TSH β 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. RXR α and RXR γ were found to be equally potent in enhancing negative regulation while RXR β was less efficient. This question has less physiological relevance since RXR β 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 TSH α 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 TR α 1 and TR β 1 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 RXR α 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.

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