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Nuclear Receptor Corepressors Activate Rather than Suppress Basal Transcription of Genes That Are Negatively Regulated by Thyroid Hormone

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A group of transcriptional cofactors referred to as corepressors (CoRs) were recently shown to play a central role in basal silencing of genes that contain positive triiodothyronine (T3) response elements. In a reciprocal manner, negatively regulated genes are stimulated by unliganded thyroid hormone receptor (TR) and repressed upon the addition of T3. We used a TRβ mutant, called P214R, which fails to interact with CoRs, to examine whether CoRs also play a role in the control of genes that are negatively regulated in response to T3. In studies of three negatively regulated genes (the pituitary thyroid-stimulating hormone α-subunit [TSHα], TSHβ, and hypothalamic thyrotropin-releasing hormone [TRH] genes), stimulation of basal promoter activity by unliganded TRβ was impaired by introducing the P214R CoR mutation. Coexpression of each of the CoRs SMRT (silencing mediator for retinoid receptors and TRs) and NCoR (nuclear receptor CoR) enhanced basal stimulation of the negatively regulated promoters in a TR-dependent manner, but this effect was not seen with the P214R TR mutant. The mechanism of CoR effects on negatively regulated promoters was explored further with a series of GAL4-TR chimeric receptors and mutants that allowed TR effects to be assessed independently of receptor interactions with DNA. These experiments revealed that, like the negative regulation of genes by wild-type TR, basal activation occurred with GAL4-TR, but not with the GAL4-P214R mutant, and was reversed by the addition of T3. These results suggest that TR interactions with negatively regulated genes may be driven through protein-protein interactions. We conclude that a subset of negatively regulated genes are controlled by a novel mechanism that involves TR-mediated recruitment and basal activation by SMRT and NCoR. Addition of T3 reverses basal activation, perhaps by dissociation of CoRs.

Thyroid hormone receptors (TRs) function as transcription factors to increase or decrease levels of gene expression. In the unliganded state, TRs and retinoic acid receptors can suppress or silence the basal activity of promoters that contain positively regulated hormone response elements (2, 6, 12). The addition of ligand reverses gene silencing and induces strong stimulation of these genes. Recently, nuclear corepressors (CoRs), termed NCoR (nuclear receptor CoR) (22, 25) and SMRT (silencing mediator for retinoid receptors and TRs) (9), were identified and were shown to mediate ligand-independent repression. The CoRs interact with the ligand binding domain (LBD) of nuclear receptors, and several mutations in the CoR box at the amino-terminal end of the LBD have been shown to disrupt interactions with CoRs (9, 22, 25).

Although most research in the thyroid hormone action field has involved pathways of positive regulation by triiodothyronine (T3), it has been estimated that nearly equal numbers of genes are repressed in response to T3 in vivo (34). However, the molecular mechanisms responsible for TR-mediated negative regulation remain poorly defined. It is notable that in many respects, the pattern of TR effects on negatively regulated genes is the mirror image of that seen with positively regulated genes. For example, ligand-independent basal activation is seen with negatively regulated genes rather than the silencing that occurs with positively regulated genes (21, 29, 39). The addition of T3 reverses basal activation and can cause repression below the original basal level, whereas the opposite is seen with positively regulated genes.

The promoters of several negatively regulated genes have been shown to be sufficient to confer T3-dependent repression (10, 23). Several of the best-studied examples involve genes in the hypothalamic-pituitary-thyroid axis that are subject to feedback inhibition by T3. These include the hypothalamic thyrotropin-releasing hormone (TRH) gene (17, 20) and the pituitary thyroid-stimulating hormone α-subunit (TSHα) (8, 27) and TSHβ (5, 7, 37, 39) genes. In general, negative regulation has been localized to the proximal regions of these promoters. However, it has not been possible to determine a consensus negative thyroid hormone-responsive element (nTRE) from these studies. In addition, it is currently unknown how unliganded TR causes basal activation of these promoters nor is there a clear understanding of how the T3-activated receptor represses transcription. In previous studies of the TSHα promoter (27), we proposed that T3-dependent repression was likely to involve protein-protein interactions between TR and other transcription factors; this proposal differed from more traditional models in which the TR binds to high-affinity target sites in the promoter.

In this study, we considered whether TR interactions with proteins such as CoRs might play a role in the control of negatively regulated genes. We analyzed the effects of a CoR mutant of TRβ, called P214R, on both positively and negatively regulated promoters. This mutant has markedly im-

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paired function in the control of both classes of genes. Unliganded TR stimulated the basal activity of negatively regulated promoters in a manner that required the association of CoRs. We propose that, in striking contrast to positively regulated genes, negatively regulated genes are stimulated by proteins previously classified as CoRs and that the addition of T3 reverses this basal activation.

MATERIALS AND METHODS

Plasmid construction and receptor mutagenesis. The plasmid TRE-TK-Luc contains two copies (indicated in uppercase type) of a palindromic TRE (5′-ga tctcAGGTCATGACCTgagatc-3′) upstream of the thymidine kinase promoter beginning at bp −109 (TK 109) in the pA3 luciferase vector (32). The plasmid TSHα-Luc contains 846 bp of the 5′-flanking sequence and 44 bp of exon I from the human glycoprotein hormone α-subunit gene in pA3-Luc (8). The human TSHβ (hTSHβ) promoter (bp −128 to +37) was prepared by PCR with TSHβ sense primer, 5′-AAGCTTGAATTCAGTATGAATTTT-3′, and TSHβ antisense primer, 5′-GAAGCTTACTTTGCATTGGTGA-3′. The TRH promoter (bp −100 to +55) was prepared by PCR with TRH sense primer, 5′-AAGCTT CCGTCAGCGCCCCTTCCCGG-3′, and TRH antisense primer, 5′-AAGCTT ATCCGCAGTCGGCAGGTCAG-3′. These fragments were verified by DNA sequencing. The reporter plasmids TSHβ-Luc and TRH-Luc were created by inserting the relevant gene fragments into the *Hin*dIII site of pA3-Luc. The expression vector pCMV-TEF (encoding thyrotroph embryonic factor) (15) was kindly provided by M. G. Rosenfeld (University of California, San Diego). The GAL4 reporter plasmid UAS-TK-Luc contains two copies of the GAL4 recognition sequence (UAS) upstream of TK 109 in pA3-Luc.

The mutant hTRβ1 cDNAs were prepared by oligonucleotide-directed mutagenesis and verified by DNA sequencing as described previously (8) (Fig. 1A). Mutant and wild-type receptor cDNAs (hTRα1, hTRα2, hTRβ1, and rat TRβ2 cDNAs) were expressed with a Rous sarcoma virus-driven expression vector (18). The double mutant P214R/P453X was made by inserting the *Pst*I-*Bgl*II fragment of the P214R mutant DNA into the P453X receptor cDNA. An artificial *Eco*RI site was introduced into the TRβ cDNA to allow insertion of an *Eco*RI fragment encompassing the LBD of TRβ (residues 174 to 461) in frame with the GAL4 DNA binding domain (DBD) in pSG424 (36). The GAL4-P214R and other TR mutants were created by exchanging appropriate restriction fragments into the GAL4-TRβ construct. The GAL4-TRα1 and GAL4-TRα2 constructs were created similarly. The GAL4-RXRα expression vector was provided by V. K. K. Chatterjee (Cambridge University, Cambridge, United Kingdom) (38). The plasmid pCMX-SMRT was provided by R. M. Evans (Salk Institute, San Diego, Calif.) (9). The plasmid pCMX-NCoR was provided by M. G. Rosenfeld (25). The numbering of the amino acid residues of TRβ is based on a consensus nomenclature (4).

Electrophoretic mobility shift assay. The DNA binding and dimerization properties of in vitro-translated TR and mutant receptors were studied with radiolabeled TREs as described previously (31). The sequences of TRE oligonucleotides were as follows: for the TRE-LAP sense oligonucleotide, 5′-agctT GACCTGACGTCAGGTCAC-3′, and for the TRE-LAP antisense oligonucleotide, 5′-tcgaGTGACCTGACGTCAGGTCA-3′. In vitro-transcribed and -translated receptors were preincubated with in vitro-translated human retinoid X receptor α (hRXRα) or with unprogrammed lysate in 20 μl of binding buffer [20 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, and 50 μg of poly(dI-dC) per ml] at room temperature for 10 min. ^{32}P-labeled TRE oligonucleotides were added, and the mixture was incubated for an additional 20 min. The protein-DNA complexes were analyzed by electrophoresis through a 5% polyacrylamide gel with 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) buffer containing 2.5% glycerol.

Tissue culture and transient-expression assays. TSA-201 cells, clones of human embryonic kidney 293 cells that stably express the simian virus 40 large T antigen (28), were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cells were plated in 12-well dishes 16 h before transfection and were transfected by the calcium phosphate method (19). Transfection reaction mixtures contained 0.5 to 1 μg of reporter plasmids together with 20 to 500 ng of the TR or 50 to 500 ng of the GAL4 expression plasmids. When TSHβ-Luc was transfected, 50 ng of the thyrotroph embryonic factor expression vector was added (15). The total amount of each receptor construct was maintained constant in each reaction by the addition of the control plasmid without receptor. After a 6-h exposure to the calcium phosphate-DNA precipitate, Optimem (GIBCO-BRL, Grand Island, N.Y.) with 4% Dowex resin-stripped fetal bovine serum was added, with or without 10 nM T3. Cells were harvested after 30 h, and luciferase activity was measured (13).

Transient-expression assays were performed in at least duplicate transfections, and the values obtained were expressed as the means ± standard errors of the means (SEM) from at least three separate experiments. Data were analyzed for statistical significance with an unpaired Student *t* test.

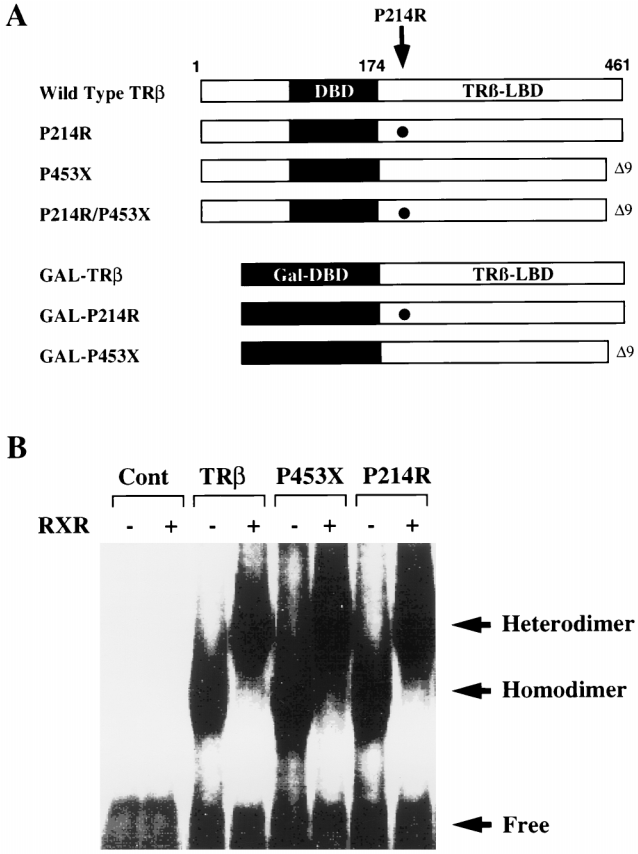

FIG. 1. Structures and DNA binding of TRβ1 mutants. (A) The full-length wild-type TRβ1 is shown. The central DBD is shaded and the carboxy-terminal LBD is indicated. The LBD of TRβ was also fused to the DBD of GAL4. Amino acid substitutions are indicated by black dots, and a nine-amino-acid carboxy-terminal-deletion mutant (P453X) is shown (Δ9). (B) DNA binding of in vitro-translated wild-type or mutant TRβ1 receptors was analyzed with ^{32}P-labeled TRE-LAP by nondenaturing gel electrophoresis. Heterodimerization was examined by adding in vitro-translated hRXRα to the preincubation mixture. The positions of heterodimers with RXR and of homodimer complexes are indicated. Cont, control.

RESULTS

Properties of a TRβ mutant (P214R) that is defective in interactions with CoRs. A mutation (P214R) was created in hTRβ that is analogous to the substitution in rat TRα1 (P160R) that was shown previously to disrupt interactions with the CoR SMRT (9). This region also corresponds to the CoR box (amino acids 211 to 240) in the hinge region of hTRβ that has been shown to interact with another CoR, NCoR (22).

The DNA and dimerization properties of the P214R mutant were examined with an everted-repeat TRE, LAP, that allows efficient binding of both homodimers and TR-RXR heterodimers (2, 30). Wild-type TRβ bound to this element as a homodimer and as a heterodimer in the presence of RXRα (Fig. 1B). The binding characteristics of the P214R and P453X mutants were indistinguishable from those of wild-type TRβ. Similar results were seen with other TRE binding sites (data not shown). These results confirm that the P214R CoR mutation does not have substantial effects on receptor homodimerization, heterodimerization with RXR, or binding to DNA.

A positively regulated thyroid hormone-responsive reporter gene (TRE-TK-Luc) was used to characterize the silencing and functional properties of TR isoforms and mutants. TSA-201

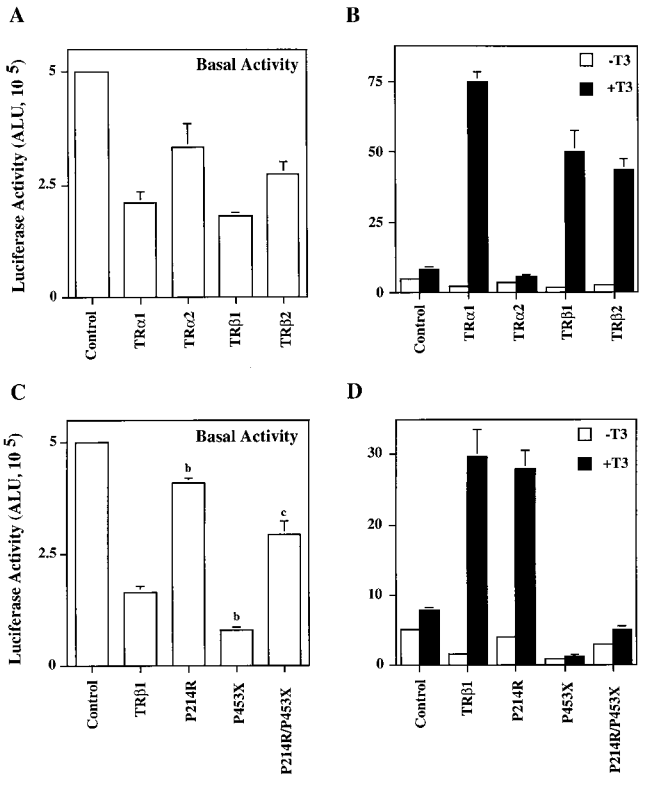

FIG. 2. Silencing activity and ligand-dependent transactivation of TR isoforms and TRβ1 mutants on a positively regulated reporter gene, TRE-TK-Luc. Wild-type or mutant receptor expression plasmids (20 ng) were transfected into TSA-201 cells together with 0.5 μg of the reporter gene TRE-TK-Luc. Silencing activity in the absence of T3 (A and C) and ligand-dependent transactivation in the presence of 10 nM T3 (B and D) were determined with a number of TR isoforms (A and B) or TRβ1 mutants (C and D). Results are the means ± SEM from three (A and B) or six (C and D) separate experiments. $P < 0.001$ versus the wild-type receptor construct (b); $P < 0.001$ versus the P453X construct (c). ALU, arbitrary light units.

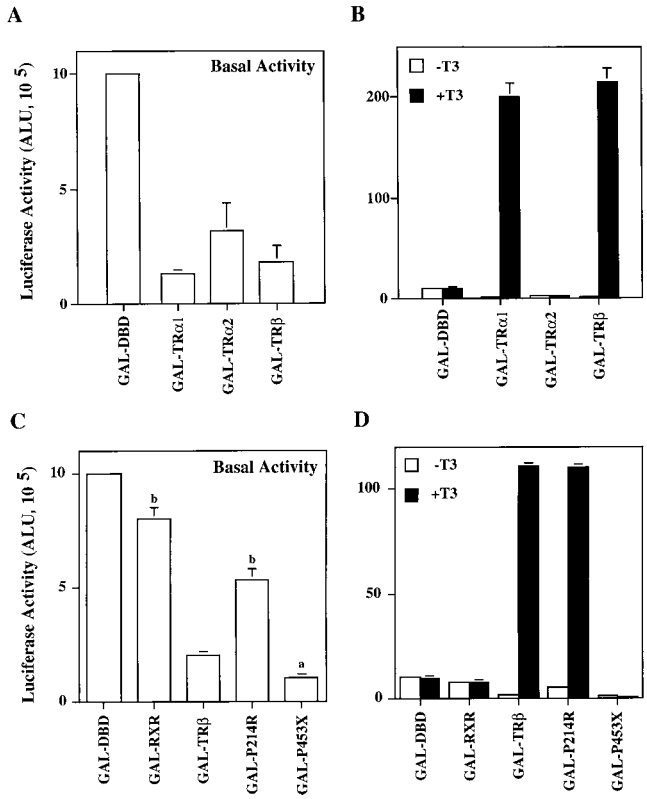

FIG. 3. Silencing activity and ligand-dependent transactivation of TR isoforms and TRβ1 mutants on a GAL4-responsive reporter gene, UAS-TK-Luc. GAL4 expression plasmids (100 ng) were transfected into TSA-201 cells together with 0.5 μg of the reporter gene UAS-TK-Luc. Silencing activity in the absence of T3 (A and C) and ligand-dependent transactivation in the presence of 10 nM T3 (B and D) were determined with a number of TR isoforms (A and B) or TRβ1 mutants (C and D). Results are the means ± SEM from three (A and B) or six (C and D) separate experiments. $P < 0.01$ (a) and $P < 0.001$ (b) versus the wild-type receptor construct. ALU, arbitrary light units.

cells, which are derivatives of 293 cells, exhibited pronounced silencing by unliganded wild-type TR (Fig. 2). Basal activity was suppressed by more than 50% with TRα1, TRβ1, and TRβ2, but there was less silencing by the non-T3 binding splicing variant, TRα2 (Fig. 2A). Addition of T3 markedly activated expression with each of the TR isoforms except TRα2 (Fig. 2B). In the absence of cotransfected TR, there was minimal T3-induced expression, confirming that this cell line is functionally deficient in TR activity.

The functional properties of the TRβ P214R mutant were also examined with TRE-TK-Luc to confirm that the mutation eliminated T3-independent silencing. In contrast to wild-type TRβ, the P214R mutant showed minimal silencing of TRE-TK-Luc in the absence of T3 ($P < 0.001$) (Fig. 2C). An additional TR mutant, P453X, contains a nine-amino-acid deletion at the carboxy terminus that includes a critical part of a transactivation domain (AF-2) (38) and eliminates T3 binding (8). This mutant showed significantly greater silencing activity than the wild-type receptor. Insertion of the P214R mutation into the background of P453X (P214R/P453X) reduced the silencing of this otherwise-potent mutant. Similar results were obtained with CV-1 cells (data not shown). T3-induced transactivation with the P214R mutant was preserved, whereas the P453X mutant was inactive in the presence of T3, which is consistent with its inability to bind the ligand (Fig. 2D).

The silencing activities of these receptor isoforms and mutants were also examined with a heterologous DBD (Fig. 3). The LBDs of the various TR isoforms were fused to the DBD of the yeast transcription factor GAL4. The reporter gene UAS-TK-Luc, which contains two GAL4 binding sites, was used to assess silencing and T3 stimulation. Relative to the GAL-DBD alone, GAL-TRα1 and GAL-TRβ induced marked silencing (Fig. 3A) (33). As with the native receptors, the silencing activity of GAL-TRα2 was somewhat reduced relative to that of the GAL-TRα1 and GAL-TRβ isoforms. T3 stimulated transactivation by GAL-TRα1 and GAL-TRβ but not GAL-TRα2 (Fig. 3B).

The silencing activity of the P214R mutant was also examined in the context of the GAL4 chimeric constructs. Whereas the TRβ LBD (GAL-TRβ) conferred strong silencing activity, introduction of the P214R mutation into the LBD abrogated silencing activity ($P < 0.001$) (Fig. 3C). As seen with the native receptor, the carboxy-terminal-deletion mutant, GAL4-P453X, potently suppressed basal activity. By comparison, GAL-RXRα exhibited minimal silencing, confirming that suppression is greater with TR than with its heterodimeric partner (9, 25). In the presence of T3, transactivation was induced to a similar extent by GAL-TRβ and GAL-P214R, indicating that the P214R CoR mutation does not impair transactivation (Fig. 3D). These results confirm that the P214R mutation markedly impairs CoR activity, with little or no effect on transactivation. In addition, the P453X mutant appears to enhance silencing,

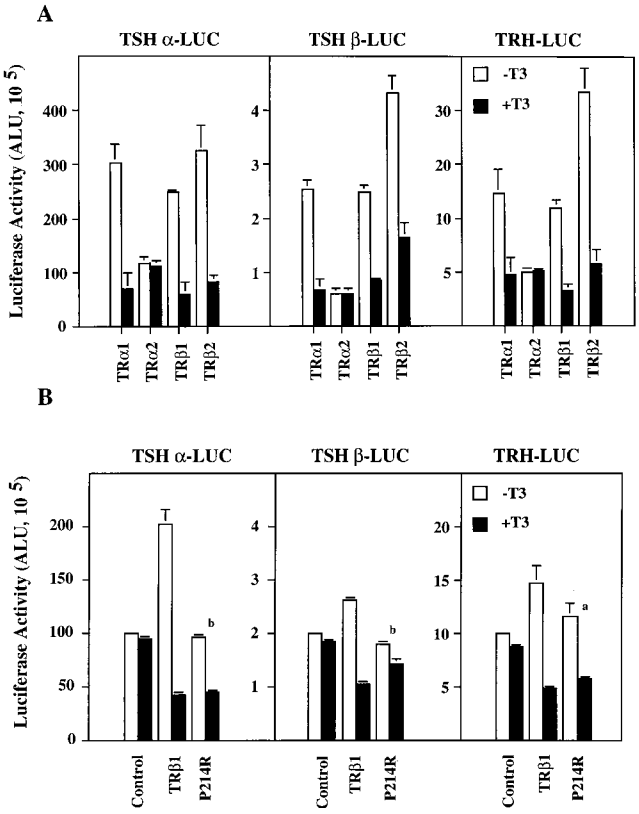

FIG. 4. The CoR mutant P214R lacks basal activation of negatively regulated genes but retains ligand-dependent repression. Wild-type or P214R expression plasmids (500 ng) were transfected into TSA-201 cells together with 1 μg of the TSHα, TSHβ, and TRH-Luc reporter genes. (A) Effects of TR isoforms on negatively regulated genes. Results are the means ± SEM from three separate experiments. (B) Effects of the P214R mutant on negatively regulated genes. Results are the means ± SEM from five separate experiments. $P < 0.05$ (a) and $P < 0.001$ (b) versus fold repression by wild-type TR. ALU, arbitrary light units.

perhaps because the AF-2 domain is missing and there is no T3 binding (T3 induces dissociation of CoR).

The TRβ CoR mutation (P214R) impairs T3-independent activation of negatively regulated genes. In contrast to positively regulated genes that are silenced by unliganded TR, there is evidence that negatively regulated genes are transcriptionally induced by unliganded TR and repressed upon the addition of T3 (21, 29, 39). A series of genes that are repressed in response to T3 (the TSHα-Luc, TSHβ-Luc, and TRH-Luc genes) were used to assess the potential role of CoRs in their control. As shown in Fig. 4A, basal stimulation by the unliganded TR was seen with each of the negatively regulated promoters. Moreover, this effect occurred with each of the TR isoforms (TRα1, TRβ1, and TRβ2) but not with TRα2. Thus, the profile of basal activation by TR isoforms with the negatively regulated promoters is the reciprocal of the phenomenon of basal silencing that was seen with the positively regulated promoter. Addition of T3 caused repression of the negatively regulated genes, which largely reflects the loss of basal activity (Fig. 4A).

The CoR mutant, P214R, eliminated the basal stimulatory activity in the absence of T3 for each the three negatively regulated promoters, TSHα-Luc, TSHβ-Luc, and TRH-Luc ($P < 0.05$) (Fig. 4B). Again, the extent of repression by T3 with the P214R mutant was significantly reduced, primarily reflecting the loss of basal activity. The effect of the P214R mutation suggests that CoRs may play a role in the basal activation of these negatively regulated genes.

CoRs enhance TR-mediated basal activation of negatively regulated genes. The loss of basal activation by the P214R mutant suggested that CoRs might be involved in basal activation. To explore this further, SMRT or NCoR constructs were cotransfected with the wild-type TRβ1 or P214R mutant plasmids with each of the three negatively regulated genes (the TSHα, TSHβ, and TRH genes) (Fig. 5). In the absence of cotransfected TRβ, the addition of SMRT or NCoR had no effect on the activity of any of the promoters of these genes. However, in the presence of wild-type TRβ, cotransfected SMRT and, to a greater degree, NCoR markedly enhanced basal activation. For TSHα, SMRT increased TR-mediated basal activation 2.2-fold, and NCoR increased basal activation 5.5-fold. For the TSHβ promoter the basal enhancement by unliganded TRβ was more pronounced, but SMRT still increased TR-mediated basal activity 1.5-fold and NCoR increased activity 2.5-fold. For the TRH promoter, SMRT increased TR-mediated basal activity 2.5-fold, and NCoR increased activity 2.9-fold. SMRT and NCoR were inactive in

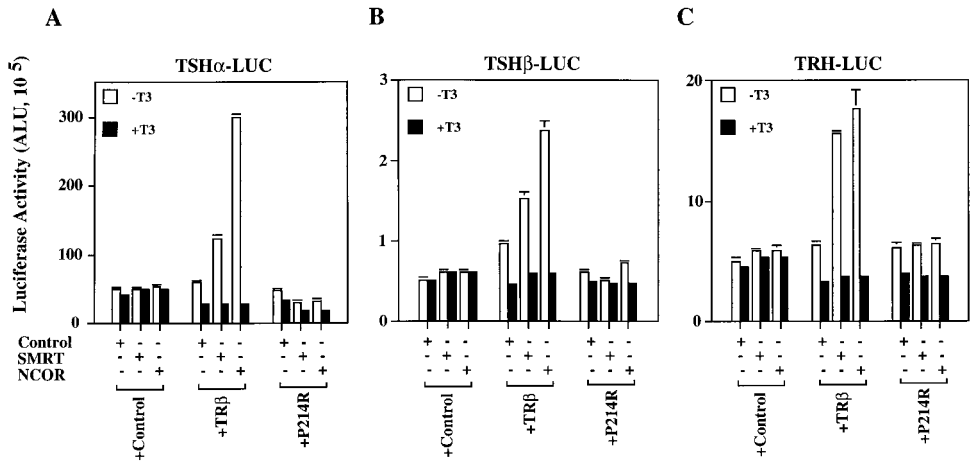

FIG. 5. Effects of exogenous CoRs on the function of negatively regulated genes. Wild-type TRβ1 or P214R expression plasmids (100 ng) were cotransfected into TSA-201 cells with control or CoR expression plasmids (400 ng) together with 1 μg of the TSHα, TSHβ, and TRH-Luc reporter genes. Cells were incubated in the absence or presence of 10 nM T3. Results are the means ± SEM from three independent experiments. ALU, arbitrary light units.

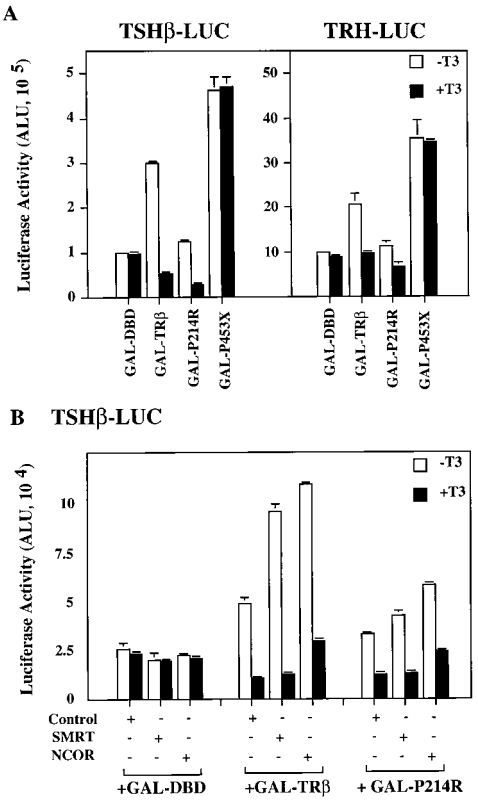

FIG. 6. GAL-TR constructs mediate the effects of the CoRs on negatively regulated promoters. (A) GAL4 expression plasmids (500 ng) were cotransfected into TSA-201 cells with 1 μg of the TSHβ-Luc and TRH-Luc reporter genes. (B) GAL4-TRβ or GAL4-P214R expression plasmids (400 ng) were cotransfected into TSA-201 cells with control or CoR expression plasmids (400 ng) together with 1 μg of TSHβ reporter genes. Cells were incubated in the absence or presence of 10 nM T3. Results are the means ± SEM from three independent experiments. ALU, arbitrary light units.

the presence of the P214R mutant, confirming that their effects are dependent upon interactions with TR. The extent of T3-induced repression was unaffected by coexpression of the CoRs, although the percentage of repression is increased by CoRs because they increase basal activity. These results indicate that CoRs might serve as TR-dependent activators in the case of negatively regulated promoters.

Evidence for protein-protein interactions in TR-mediated control of negatively regulated genes. GAL-TR constructs were used to assess whether TR-mediated effects on the basal activity of negatively regulated genes could occur in the absence of direct binding of TR to DNA. As shown in Fig. 6, the effects of GAL-TRβ were similar to those observed with wild-type receptor. GAL-TRβ stimulated basal expression of the TSHβ and TRH promoters in the absence of T3 and induced repression upon the addition of T3. Similar to the native receptors, the P214R mutant lacked basal activation, although weak T3-induced repression was still seen. The GAL-P453X mutant led to marked basal activation that was not reversed after the addition of T3. Similar results were also seen with the LBD of TR in the absence of the GAL4 DBD (data not shown).

At least two mechanisms might account for the ability of GAL-TR to stimulate the basal activity of these promoters and for the ability of T3 to reverse this effect: (i) GAL-TR could compete for repressors, and these could be dissociated upon T3 binding; or (ii) GAL-TR might associate with promoter-bound factors and recruit SMRT or NCoR, which could function as activators. To distinguish these possibilities, SMRT and NCoR were cotransfected with the expectation that competition via the first mechanism might be reversed by excess protein whereas recruitment via the second mechanism might be enhanced. As shown in Fig. 6B, cotransfection of excess SMRT or NCoR with GAL-TRβ enhanced basal activation, which is analogous to the effect obtained with wild-type TRβ. With the GAL-P214R mutant, the effects of SMRT and NCoR were greatly reduced. There was no evidence for competition for a limiting amount of RXR, as cotransfection of RXR further enhanced, rather than reversed, basal activation (data not shown). These findings argue against a mechanism in which GAL-TR competes for CoRs or other limiting factors and suggest that proteins previously classified as CoRs can also function as activators in the context of negatively regulated promoters.

DISCUSSION

A role for CoRs in negative regulation initially seems counterintuitive, as these proteins have previously been studied primarily as factors that repress positively regulated genes in the absence of hormone. However, we have demonstrated that TR-mediated effects on negatively regulated promoters, such as those derived from the TSHα, TSHβ, and TRH genes, are impaired by a mutation (P214R) that prevents effective TR-CoR interactions. In particular, this TR mutant prevents the basal-level activation of these promoters by unliganded TRβ. Cotransfection with either of the CoRs SMRT and NCoR enhanced TR-dependent basal activation of negative promoters, and this effect was not seen after insertion of the P214R mutant. These results suggest that a CoR(s) participates in the control of negatively regulated genes, particularly with respect to basal activation by the unliganded receptor.

The interpretation of our studies depends a great deal upon the properties of the TRβ P214R mutation. The P214R mutant (with a Pro-to-Arg substitution) is homologous to the avian erythroblastosis virus (v-erbA) transformation-deficient mutant td359, which is deficient in the silencing of thyroid hormone-responsive genes and in the transformation of erythroblasts (11). In fact, we originally prepared this TRβ mutant based upon these properties of the v-erbA mutant. The P214R mutant is also the hTRβ homolog of the rat TRα1 mutant P160R, which is unable to interact with SMRT (9). NCoR has also been reported to bind to this region of hTRβ (CoR box, residues 211 to 240) (22). Thus, it is likely that this mutation disrupts interactions with several different CoRs and perhaps other proteins that have not been identified. In addition to the previous studies of TR-CoR interactions noted above, our own results validate the idea that the P214R mutant disrupts interactions with CoRs. Insertion of this mutant into full-length hTRβ (Fig. 2C) or into the fusion protein GAL4-P214R (Fig. 3C) resulted in the loss of transcriptional silencing of a positively regulated promoter by the unliganded receptor. Since the P214R mutant retained T3-induced activation of these positively regulated promoters, this mutant appears to be selectively defective in the binding of CoRs but to retain the ability to interact with coactivators (CoAs). Based upon gel shift studies (Fig. 1B) and interactions with RXR–VP-16 (data not shown), it also appears to retain normal DNA binding and dimerization with RXR.

One of the mutants that we studied, P453X, demonstrated enhanced silencing of positively regulated promoters and caused increased basal-level stimulation of negatively regulated genes. This mutant contains a nine-amino-acid carboxy-

terminal deletion which eliminates a transactivation domain termed AF-2 (3, 16) or tau4 (1), and it does not bind T3. This deletion corresponds to one of the alterations in v-erbA, which is also a potent silencer of T3-responsive genes (11, 12, 35, 40). T3 causes the dissociation of CoRs and seems to induce a conformational change in the AF-2 domain that facilitates the association of one or more CoAs (1, 9, 24, 26, 38). It is likely that this carboxy-terminal deletion causes two separate effects to prevent ligand binding and to eliminate the AF-2 domain. It has been shown that deletion of only the carboxy-terminal six amino acids results in a receptor that still binds hormone but acts as a constitutive silencer (1), raising the possibility that the extreme C terminus is required for T3-induced dissociation of CoRs from the receptor. It is possible, therefore, that interactions of CoRs and CoAs with TR are mutually exclusive.

The understanding of how CoRs control thyroid hormone-responsive genes is still evolving. In the case of positively regulated genes, CoRs have been proposed to bind to TR in the absence of T3 and to silence basal transcription (9, 22, 25). Upon addition of T3, CoR dissociates from TR and relieves silencing. In addition, transcriptional CoAs are recruited to the T3-bound TR. Our results are consistent with this model. However, there is much less information about mechanisms that control negatively regulated genes. Like previous studies, the present study showed that unliganded TR causes basal activation of negatively regulated genes and that this is reversed upon the addition of T3 (Fig. 4A). Thus, the negative regulation appears to be largely a consequence of "reversal of activation" rather than active suppression of promoter activity by T3. In fact, it is remarkable that the TR effects on the three negatively regulated promoters examined in this study are essentially a mirror image of the effects seen with positively regulated genes. A role for CoRs in the basal activation of the negatively regulated genes is implied by the lack of stimulation by the P214R mutant (Fig. 4B).

We initially postulated a partitioning model for negative regulation in which basal activation might be accounted for by TR competition for CoRs bound to other factors on the promoter. In this manner, TR might cause basal activation by relieving repression. Although a partitioning model cannot be entirely excluded, the experiments in which excess CoRs were cotransfected argue against such a mechanism. For example, coexpression of SMRT or NCoR enhanced, rather than reversed, basal activation by TR (Fig. 5B). In a partitioning mechanism, one would predict excess CoR to saturate the competitor TR and allow interaction and silencing of other promoter-bound proteins. It is notable, however, that the stimulatory effects of SMRT and NCoR are dependent upon interactions with the TR, as they were not observed in the case of the P214R mutant.

As an alternative, we considered the paradoxical possibility that CoRs might be able to function as activators in the context of the negatively regulated promoters. As noted above, addition of SMRT and NCoR enhanced TR-mediated basal activation of the negatively regulated promoters. These effects are not readily attributable to the cell context or other experimental parameters because the CoRs silenced the positively regulated promoter under the same conditions (data not shown). These data suggest, therefore, that SMRT and NCoR can serve as either TR-dependent activators or repressors, depending upon the promoters that are targets of TR action.

The possibility that protein-protein interactions may play a role in the control of negatively regulated genes is suggested by the effects of the non-DNA binding construct GAL-TR. The effects of GAL-TR and GAL-P214R were nearly identical to those seen with their full-length receptor counterparts. Similar results were seen with constructs that express only the LBD of the TR (data not shown). For unclear reasons, mutants with site-directed substitutions in the TR DBD failed to show this effect, but it is possible that these mutants assume an altered conformation that precludes interactions with CoRs. As with the native receptors, coexpression of SMRT or NCoR enhanced rather than reversed the basal activation that occurred with GAL-TR. In view of these results, we tentatively favor a model in which protein-protein interactions recruit TR to the promoters of negatively regulated genes and in which proteins, such as SMRT or NCoR, that were previously classified as repressors serve as activators. However, given the complexity of the system, this model merits further evaluation with additional receptor mutants and ultimately promoter factors that are targets for TR.

One of the implications of these studies is that direct interactions of the TR with promoter regulatory elements may not be required to control negatively regulated genes. This raises the question of how specific responses are achieved, and several explanations might be considered. These data do not exclude TR interactions at other sites in these genes but only demonstrate that such interactions are not mandatory under the conditions employed. Thus, other TR binding sites that serve to localize the TR to specific genes could exist. Another possibility is that composite enhancers, analogous to the glucocorticoid–AP-1 element in the proliferin promoter (14), could interact with TR in a manner to attract the TR to a sequence that would otherwise not represent a consensus TRE binding site. It is notable that each of the negatively regulated promoters examined in this study has been shown to contain regulatory elements for AP-1 or CREB. These proteins may therefore represent direct or indirect (e.g., via SRC or CREB binding protein) targets for TR.

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