Transcriptional Silencing by Unliganded Thyroid Hormone Receptor β Requires a Soluble Corepressor That Interacts with the Ligand-Binding Domain of the Receptor

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Unliganded thyroid hormone receptor (TR) functions as a transcriptional repressor of genes bearing thyroid hormone response elements in their promoters. Binding of hormonal ligand to the receptor releases the transcriptional silencing and leads to gene activation. Previous studies showed that the silencing activity of TR is located within the C-terminal ligand-binding domain (LBD) of the receptor. To dissect the role of the LBD in receptor-mediated silencing, we used a cell-free transcription system containing HeLa nuclear extracts in which exogenously added unliganded TR β repressed the basal level of RNA polymerase II-driven transcription from a thyroid hormone response element-linked template. We designed competition experiments with a peptide fragment containing the entire LBD (positions 145 to 456) of TR β . This peptide, which lacks the DNA-binding domain, did not affect basal RNA synthesis from the thyroid hormone response element-linked promoter when added to a cell-free transcription reaction mixture. However, the addition of the LBD peptide to a reaction mixture containing TR β led to a complete reversal of receptor-mediated transcriptional silencing in the absence of thyroid hormone. An LBD peptide harboring point mutations, which severely impair receptor dimerization, also inhibited efficiently the silencing activity of TR, indicating that the relief of repression by the LBD was not due to the sequestration of TR or its heterodimeric partner retinoid X receptor into inactive homo- or heterodimers. We postulate that the LBD peptide competed with TR for a regulatory molecule, termed a corepressor, that exists in the HeLa nuclear extracts and is essential for efficient receptor-mediated gene repression. We have identified the region from positions 145 to 260 (the D domain) of the LBD as a potential binding site of the putative corepressor. We observed further that a peptide containing the LBD of retinoic acid receptor (RAR) competed for TR-mediated silencing, suggesting that the RAR LBD may bind to the same corepressor activity as the TR LBD. Interestingly, the RAR LBD complexed with its cognate ligand, all-*trans* retinoic acid, failed to compete for transcriptional silencing by TR β , indicating that the association of the LBD with the corepressor is ligand dependent. Finally, we provide strong biochemical evidence supporting the existence of the corepressor activity in the HeLa nuclear extracts. Our studies demonstrated that the silencing activity of TR was greatly reduced in the nuclear extracts preincubated with immobilized, hormone-free glutathione *S*-transferase-LBD fusion proteins, indicating that the corepressor activity was depleted from these extracts through protein-protein interactions with the LBD. Similar treatment with immobilized, hormone-bound glutathione *S*-transferase-LBD, on the other hand, failed to deplete the corepressor activity from the nuclear extracts, indicating that ligand binding to the LBD disrupts its interaction with the corepressor. From these results, we propose that a corepressor binds to the LBD of unliganded TR and critically influences the interaction of the receptor with the basal transcription machinery to promote silencing. Ligand binding to TR results in the release of the corepressor from the LBD and triggers the reversal of silencing by allowing the events leading to gene activation to proceed.

The thyroid hormone receptors (TRs), TR α and TR β , belong to the steroid-thyroid receptor superfamily of ligand-inducible transcription factors (15, 19). TRs regulate transcription of specific cellular genes harboring thyroid hormone response elements (TREs) in their promoters (31, 43). It is thought that in the cell, TR binds to the TRE in the target gene promoter in a ligand-independent manner. In vitro studies show that TR may bind to a TRE as a monomer, a TR-TR homodimer, or a heterodimer of TR with related nuclear hormone receptors such as the retinoid X receptor (RXR) (12, 21, 30, 36, 39, 40, 46, 48). TRE-bound TR exhibits dual gene-regulatory properties and may function as either a transcriptional activator or a repressor depending on the hormonal

status of the receptor (17, 25). Previous studies demonstrated that hormone-free TR α or TR β or a non-hormone-binding oncogenic variant of TR α , v-erbA, actively silences basal transcription of TRE-linked promoters (5, 7, 9, 17, 20, 42, 45). Binding of hormonal ligand to TR triggers the release of transcriptional silencing and leads to activation of target gene expression (17, 20, 45).

To investigate the molecular basis of TR-mediated gene silencing and its hormone-induced reversal, we performed cell-free reconstitution experiments with purified human TR β , HeLa cell nuclear extracts, and a reporter gene containing a minimal TATA promoter linked to TREs (45). Addition of unliganded TR β to this transcription system repressed basal transcription from the TRE-linked promoter. Binding of thyroid hormone to the receptor reversed this transcriptional silencing. Specific binding of TR β to the TRE in the target promoter was crucial for silencing. Studies with truncation mutants of TR β indicated that the silencing activity is located

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within the C-terminal rather than the N-terminal domain of the receptor (45). Previous transient-transfection studies by Baniahmad et al. demonstrated that the silencing function of TR β is located within the C-terminal LBD between amino acid residues 168 and 456 (4). Fusion of this fragment of TR β with the DNA-binding domain of an unrelated transcription factor Gal4 resulted in a chimeric protein that bound to a Gal4 target promoter and displayed potent silencing function. These results suggested that the silencing function can be uncoupled from the native DNA-binding domain, as well as the specific hormone response element.

To analyze how the C-terminal ligand-binding domain (LBD) of TR mediates gene repression, we performed competition experiments in which bacterially synthesized peptides containing the LBD of either human TR β (hTR β) or human retinoic acid receptor α (hRAR α) were used as competitors of silencing activity of the wild-type TR β in cell-free transcription extracts. We observed that the TR LBD fragment, which did not affect the binding of TR to its hormone response element, efficiently suppressed the TR-mediated transcriptional silencing in HeLa nuclear extracts in the absence of hormonal ligand. The RAR LBD peptide also suppressed gene silencing by TR. More interestingly, binding of retinoic acid to the RAR LBD prevented such inhibition of silencing. We present evidence that ligand-free LBD of TR β or RAR α binds to a limiting soluble regulatory cofactor, which exists in the HeLa nuclear extracts, through protein-protein interactions. We propose that this is the basis of competition for the silencing function of TR β by the LBD peptides. Ligand-bound LBD, on the other hand, does not interact with this cofactor and therefore fails to compete for silencing by unliganded TR. Our results therefore suggest a critical modulatory role for a corepressor activity in TR-mediated transcriptional silencing and its hormone-induced reversal.

MATERIALS AND METHODS

Plasmids. The construction of the transcription templates TRE₂TATA and pAdML200 has been described previously (2, 3, 45).

Bacterial expression and purification of TR β . A full-length cDNA of human TR β with 5' *Nde*I and 3' *Bam*HI ends was introduced between the *Nde*I and *Bam*HI sites of the bacterial expression vector pET15b (Novagen, Inc., Madison, Wis.). The expression of TR β as a hexahistidine (linked to the amino terminus of the receptor) fusion protein in *Escherichia coli* and its purification by nickel affinity chromatography were carried out by previously published procedures (29, 45). Control fractions containing bacterial proteins only were generated from *E. coli* transformed with empty pET15b vector by following the same protocol. The Ni-affinity-purified TR β preparations were typically 50 to 70% pure as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Bacterial expression and purification of human RXR α were performed as described previously (45).

Cell-free transcription assay. The cell-free transcription conditions and isolation of ³²P-labeled transcripts have been described previously (2, 3). In competition experiments, the reactions were carried out in three stages. In the first stage, HeLa nuclear extracts were incubated at 30°C for 15 min with or without hTR β and/or the TR β LBD or RAR α LBD peptide. The incubations were performed either in the presence or in the absence of 10⁻⁶ M [4-(4-hydroxy-3-iodophenoxy)-3,5-diiodophenyl]acetic acid (Tria) or 10⁻⁶ M all-*trans* retinoic acid. In the second stage, template DNAs were added and the incubations were continued at 30°C for 15 min. In the third stage, other transcription reaction components and nucleoside triphosphates were added and the RNA syntheses were continued for 45 min at 30°C.

Electrophoretic mobility shift assay. The electrophoretic mobility shift assays were performed as described previously (45).

Construction of TR β LBD and its mutants. The LBD fragments containing the regions from positions 145 to 456 and 145 to 260 of human TR β , respectively, were constructed by PCR-assisted and oligonucleotide-directed mutagenesis. The resulting DNA fragments, containing 5' *Nde*I and 3' *Bam*HI ends, were subcloned into pET15b expression vector. The RAR α LBD fragment containing the region from 136 to 462 of human RAR α and flanked by 5' *Nde*I and 3' *Bam*HI ends was constructed and incorporated into the bacterial expression vector pET15b by a similar procedure. The LBD peptides of TR β and RAR α were expressed in *E. coli* as hexahistidine fusion proteins and purified by nickel affinity chromatography as described previously (29, 45). The TR β LBD peptide

from 145 to 456 and the RAR α LBD peptide from 136 to 462 displayed ligand-binding affinity (K_d = 1 nM) similar to that of the corresponding full-length receptor.

The mutant TR β _{415,416} containing two point mutations, lysine to glutamic acid at position 415 and leucine to phenylalanine at position 416, was constructed by creating an *Eco*RI site in the LBD region by PCR-assisted and oligonucleotide-directed mutagenesis. The wild-type nucleotide sequence -CCA AAA CTC CTG-, coding for amino acids -PKLL-, was changed to the sequence -CCA GAA TTC CTG-, coding for amino acids -PEFL. The LBD (positions 145 to 456) corresponding to this mutant TR β was synthesized as described above. The mutant TR β and the corresponding LBD peptide were expressed in *E. coli* and purified by Ni affinity chromatography following standard protocols.

Treatment of HeLa extracts with immobilized LBD peptides. A cDNA fragment coding for the TR β LBD (positions 145 to 456) was engineered into plasmid pGEX-2T (Pharmacia), and the LBD was expressed as a fusion protein with glutathione *S*-transferase (GST). The expressed GST-LBD was purified from bacterial extracts by binding to glutathione affinity resin. The GST-LBD bound to 3,5,3'-triiodothyronine (T₃) with an affinity similar to that of the full-length TR β . The bound resin was incubated with or without thyroid hormone (1 μ M) and was washed extensively with a buffer containing 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.9), 100 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 5 mM magnesium chloride, and 20% glycerol. HeLa nuclear extracts (250 to 300 μ g) were incubated with GSH-affinity resin, bound to 1 μ g of GST, GST-LBD, or T₃-GST-LBD, at 4°C for 1 h with mild agitation. The resin was separated from the extract by brief centrifugation. The resulting extracts were then assayed for their ability to support TR-mediated silencing as described in Results.

RESULTS

A TR β LBD peptide suppresses receptor-mediated transcriptional silencing in a cell-free transcription system. We described previously the development of a cell-free transcription system in which addition of bacterially synthesized human TR β regulated RNA synthesis from a minimal promoter, containing a TATA box and two copies of a palindromic TRE_{pal} or a direct-repeat TRE_{DR+4}, in a thyroid hormone-dependent manner (45). As shown in Fig. 1, addition of unliganded TR β (1 pmol) to HeLa nuclear extracts repressed more than 80% of basal-level transcription by RNA polymerase II from the test template containing TREs (compare lanes 1 and 2). Binding of the hormonal ligand to TR β completely reversed this transcriptional repression (lane 3). We have shown previously that the TR-mediated silencing is entirely TRE dependent, since the response could be abolished by deletion of the TREs in the target promoter (45).

To examine how the C-terminal LBD of TR β modulates silencing, we designed competition experiments involving cell-free transcription reactions with a peptide harboring the entire LBD of TR β but lacking the DNA-binding domain. The LBD peptide containing the region from 145 to 456 of TR β was expressed in *E. coli* as a histidine-tagged protein and was purified by nickel affinity chromatography. Addition of this peptide (10 pmol) alone to the HeLa nuclear extracts did not significantly affect basal RNA synthesis from the TRE-linked (test) promoter or an adenovirus major late (internal control) promoter, either in the presence or in the absence of hormone (Fig. 1, compare lane 1 with lanes 8 and 9). Surprisingly, however, the addition of the LBD peptide to a transcription reaction mixture containing TR β reversed completely the silencing activity of the receptor in the absence of thyroid hormone (lanes 4 and 6). When the peptide was added to a transcription reaction mixture containing TR β in the presence of thyroid hormone, an additional modest enhancement of RNA synthesis above basal level was observed (lanes 5 and 7). These results demonstrated that the LBD fragment from 145 to 456, which does not interact directly with the TRE, could efficiently suppress transcriptional silencing by full-length TR β in the absence of hormone.

We next investigated whether the LBD peptide reversed TR-mediated transcriptional silencing in a dose-dependent

FIG. 1. The LBD of TR β suppresses receptor-mediated transcriptional silencing in nuclear extracts. The linear structures of human TR β and the LBD peptide are shown above the gel. Purified TR β (1 pmol) and TR β LBD peptide from 145 to 456 (8 pmol) were added as indicated to a cell-free transcription reaction mixture containing HeLa nuclear extracts and a TRE_{pal}-linked reporter template, TRE₂TATA. Triac, a thyroid hormone analog, was added at a final concentration of 1 μ M, where indicated. The transcription reactions (each in a volume of 30 μ l) were carried out, and the transcripts were analyzed as described previously (2, 3). The solid and open arrowheads indicate transcripts from the TRE_{pal} promoter and the internal control AdML promoter, respectively. The variation in the AdML signal among the lanes was due to incomplete recovery and/or unequal loading of the RNA samples. Quantitation of the RNA signals generated by the test template was performed by densitometry followed by normalization with respect to the internal-control AdML signals and is shown below the gel. The value for basal transcription (lane 1) was set to 100%, and the corresponding values for test measurements (lanes 2 to 9) were obtained. Three independent sets of the same experiment were performed, and the results of a representative experiment are shown. The standard error of the mean values ranged between 1 and 5%.

manner. Our results showed that the addition of increasing amounts of the LBD peptide (3 to 10 pmol) to the transcription reaction mixture containing TR β (1 pmol) resulted in a progressive inhibition of receptor-mediated silencing of the

TRE-linked promoter (Fig. 2A). By our estimate, addition of a six- to eightfold molar excess of the LBD peptide could efficiently reverse silencing by TR β (Fig. 2A, lanes 4 and 5). Since our receptor preparations were 50 to 70% pure, we examined whether contaminating bacterial proteins present in our receptor preparations had any effect on RNA synthesis. We observed that the addition of equivalent amounts of control bacterial proteins (see Materials and Methods) to the HeLa nuclear extracts did not significantly affect the basal level of transcription (Fig. 2B, compare lane 1 with lane 6, 7, or 8). The addition of the bacterial proteins in the presence of TR β also failed to mimic the reversal of transcriptional silencing by the LBD peptide (Fig. 2B, lanes 3 to 5), demonstrating the specificity of this phenomenon.

The LBD fragment (positions 145 to 456) by itself did not repress basal RNA synthesis (Fig. 2A, lanes 6 to 8). Therefore, any direct, functional interaction between this peptide fragment and the components of the basal transcription machinery under the conditions of our transcription reactions appears rather unlikely. We postulate the following scenario to explain our results. A soluble cofactor exists in limiting amounts in the HeLa nuclear extracts. This cofactor, termed a corepressor, interacts with the LBD of TR β , presumably through protein-protein interactions, and is critical for the silencing function of the receptor. When an excess of the LBD peptide (positions 145 to 456) is added to a transcription reaction, it binds to this limiting corepressor and sequesters this regulatory molecule, so that its interaction with TR is prevented and silencing is blocked. According to this hypothesis, a competition between the full-length receptor and the LBD peptide for the corepressor leads to the reversal of transcriptional silencing by unliganded TR.

TR β LBD does not inhibit DNA binding by TR-RXR heterodimers. Studies by Samuels and coworkers have indicated that conserved heptad repeat motifs containing leucines in the LBD of TR or RAR constitute a signal for receptor dimerization (1, 22, 23). It is therefore conceivable that the TR β LBD fragment from 145 to 456, when added to a transcription reaction mixture containing the full-length receptor and HeLa nuclear extracts, may generate either homodimers with TR or heterodimers with the endogenous RXRs in the HeLa nuclear extracts. Such interactions may lead to the inhibition of DNA binding of the unliganded receptor through sequestration of TR or RXR into inactive homo- or heterodimers and could be the basis of the suppression of TR-mediated silencing by the LBD peptide (23). We observed that most of the exogenously added TR β , when combined with HeLa transcriptional extracts, did not bind to the target DNA either as monomer or as a homodimer but formed exclusively heterodimers with the endogenous pool of RXRs or other T₃ receptor auxiliary proteins (TRAPs) in the nuclear extracts (data not shown). Similar results have been reported by other laboratories (36). To test whether the LBD peptide can interfere with the DNA binding of the TR-RXR heterodimers, we performed gel shift analyses as described for Fig. 3. A combination of purified TR β and RXR α readily formed a heterodimer that bound to DNA efficiently. The addition of excess LBD peptide to this reaction mixture failed to inhibit DNA binding by TR-RXR heterodimers even at a molar ratio of LBD to TR of 20:1. These results suggested that it is unlikely that the reversal of TR-mediated silencing by the LBD peptide in HeLa nuclear extracts is due to an inhibition of DNA binding of the unliganded receptor.

A dimerization-defective LBD suppresses TR-mediated silencing. We addressed the issue of a possible role of the dimerization activity of the LBD peptide in the reversal of TR-

FIG. 2. The LBD peptide suppresses TR-mediated transcriptional silencing in a dose-dependent manner. (A) Increasing amounts of TR β LBD from 145 to 456 (3.0, 5.5, and 8.0 pmol) were added to the cell-free transcription reaction mixtures in the presence or absence of TR β (1 pmol). The concentration of TR β was determined by binding to [125 I]T $_3$. The concentration of the LBD peptide was determined by comparing the intensity of the stained peptide band with that of TR β in SDS-PAGE. The RNA transcripts generated from the test template were quantitated by densitometry and normalized with respect to the AdML transcripts. The results are shown below the gel. The value of the basal transcription (lane 1) was adjusted to 100%. Three independent sets of the same experiment were performed, and the results of a representative experiment are shown. (B) Increasing amounts of bacterial proteins (0.18, 0.33, and 0.48 μ g) were added to cell-free transcription reaction mixtures. The doses used in these reactions were equivalent in protein content to the doses used in the experiment in panel A. The protein amounts were determined from the facts that (i) 1 pmol of pure LBD corresponds to 30 ng protein and (ii) the LBD preparations were approximately 50% pure. Quantitation of RNA signals was performed as described for panel A. The results are shown below the gel. Three independent sets of the same experiment were performed, and the results of a representative experiment are shown. The standard error of the mean values ranged between 1 to 5%.

mediated silencing by designing a more stringent experiment. For this purpose, we first created a dimerization-defective mutant of TR β by altering the amino acid residues within the conserved ninth heptad in the LBD. In this mutant TR $\beta_{415,416}$, lysine at position 415 was changed to glutamic acid and leucine at position 416 was changed to phenylalanine. This mutant receptor was expressed in *E. coli*, and the purified protein was tested for its dimerization and DNA-binding activities. As shown in the gel shift experiments in Fig. 4, the bacterially produced wild-type TR β bound only weakly to a 32 P-labeled TRE $_{\text{pal}}$ probe and formed TR-TR homodimers. Whereas the wild-type TR β readily formed TR-RXR heterodimers in the presence of RXR α , the mutant TR β failed to generate either homo- or heterodimers on a TRE probe. The mutant TR $\beta_{415,416}$ was therefore completely devoid of any detectable dimerization activity.

We expressed the LBD peptide corresponding to the region from 145 to 456 of the mutant TR $\beta_{415,416}$ in *E. coli* and purified it by affinity chromatography. We then used the mutant TR β LBD $_{415,416}$ in competition experiments (Fig. 5). Our results showed that the addition of increasing amounts of the dimerization-defective LBD $_{415,416}$, like the wild-type LBD, progressively reversed TR-mediated silencing in the absence of hormone. Since the mutant LBD peptide is not capable of forming any homo- or heterodimer with TR or RXR, the results of this experiment suggested strongly that the LBD of TR β bound

and titrated out a soluble cofactor (corepressor) that is likely to be distinct from the receptor itself or RXR. This result also indicated that heterodimerization with RXR is not a prerequisite for the association of the TR LBD with the corepressor activity.

Identification of a corepressor-binding site within the TR β LBD. To define precisely the region within the LBD that interacts with the corepressor, we designed competition experiments in the cell-free transcription system by using shorter LBD peptides. When we attempted to express peptides spanning various regions within the LBD (positions 145 to 456) in *E. coli*, we had only mixed success. We found that a peptide from 145 to 260 was expressed abundantly in *E. coli* whereas another peptide from 260 to 456 was expressed rather poorly and was presumably unstable in the bacteria. When we used the peptide from positions 145 to 260 in competition experiments, as shown in Fig. 6A, addition of this peptide alone (up to 30 pmol) to HeLa nuclear extracts did not affect the basal level of transcription. We observed, however, that the addition of this peptide to a transcription reaction mixture containing TR β (1 pmol) fully reversed silencing in the absence of hormone. We then performed an experiment in which increasing concentrations of the peptide from 145 to 260 were added to cell-free transcription reaction mixtures containing unliganded TR β (Fig. 6B). We noted that approximately a 25-fold molar excess (relative to TR β) of this peptide was required to com-

FIG. 3. The LBD peptide does not interfere with TR-RXR heterodimer formation on a TRE_{pal}. A ³²P-labeled double-stranded oligonucleotide (20 bp long, 0.1 ng) containing TRE_{pal} sequence was used as a probe in the electrophoretic mobility shift assay, which was performed as described previously (45). TR β (0.5 pmol), RXR α (1.5 pmol), and the LBD peptide (5 pmol for lane 6 and 10 pmol for lanes 2, 4, and 7) were combined with the DNA probe as indicated. TR-RXR represents the heterodimeric complex of TR β and RXR α .

pletely reverse receptor-mediated transcriptional silencing. This molar ratio (peptide/TR β) is significantly higher (approximately threefold) than that of the larger LBD fragment from 145 to 456, which is required to achieve a similar reversal effect. Nevertheless, the ability of the peptide from 145 to 260 to relieve transcriptional repression by TR β indicated that this peptide alone was sufficient to titrate out the soluble negative effector present in the HeLa nuclear extracts. We have therefore located a potential corepressor-binding site within a stretch of 115 amino acids in the LBD. This sequence encompasses the D domain (positions 170 to 238) and the N-terminal region (positions 238 to 260) of the E domain of TR β (15, 38). Interestingly, our results are consistent with those of Banihmad et al., who have shown recently by transient-cotransfection assays that the region from 168 to 259 of the LBD of TR β competed for the silencing activity of the receptor (6).

Silencing by unliganded TR is suppressed by a RAR LBD peptide. Previous studies showed that the RAR, like TR, displays transcriptional silencing activity in the unliganded state (5, 13). We therefore examined whether the LBD of RAR can interfere with TR-mediated silencing in cell-free transcription experiments. For this purpose, we expressed in *E. coli* a peptide containing the entire ligand-binding region (from 136 to 462) of RAR α . The addition of the RAR LBD alone to a transcription reaction mixture did not affect basal RNA synthesis from a TRE-containing promoter (Fig. 7, lanes 5 and 6). However, when we combined the RAR LBD peptide (15 pmol) with TR β (1 pmol) in competition experiments, we observed that the TR-mediated repression of basal transcription in the absence of ligand was reversed entirely upon addition of this peptide (lane 4). The effect of the RAR LBD was also dose dependent, since addition of 7.5 pmol of the peptide had only marginal effect on silencing (lane 3). These results indicated that both TR LBD and RAR LBD interact with and therefore compete for a common corepressor that is required for TR- or RAR-mediated transcriptional silencing.

Ligand binding to the RAR LBD prevents competition for TR-mediated silencing. Transcriptional silencing by TR or RAR is reversed by the cognate hormone (5, 17, 20, 45). Ligand binding is also known to induce an alteration in the conformation of the LBD (37, 44). It is therefore of interest to investigate whether the binding of the hormonal ligand to the TR or RAR LBD influences its interaction with the putative corepressor. The effect of thyroid hormone on the competition between TR β and TR β LBD is difficult to interpret, because both species bind to the hormone. For this reason, we used RAR LBD peptides to compete for TR β -mediated silencing either in the presence or in the absence of all-*trans* retinoic acid. We have already shown that hormone-free RAR LBD peptide competes with TR β for a limiting amount of corepressor (Fig. 7). Addition of all-*trans* retinoic acid to this system will not affect the interaction between TR β and the corepressor. The RAR LBD peptide, however, binds to this ligand with an affinity that is similar to that of the full-length RAR (see Materials and Methods). If ligand binding to this peptide affects its interaction with the corepressor, it will be reflected in the competition assay. The results presented in Fig. 8 clearly indicate that the addition of retinoic acid did indeed influence the ability of the RAR-LBD to compete for TR-mediated silencing. Whereas the unliganded RAR LBD peptide reversed efficiently the silencing of basal transcription by TR β , the ligand-bound RAR LBD failed to suppress such repression (Fig. 8, compare lanes 3 and 4). It is likely that ligand binding to RAR-LBD impaired its ability to interact with the limiting corepressor, which remained available for binding to the repressor, TR β , to effect silencing. Our results suggest strongly that the association of the RAR LBD with the corepressor is ligand dependent.

Depletion of the corepressor activity from HeLa nuclear extracts impairs TR-mediated transcriptional silencing. The observation that the TR β LBD (145-456) peptide competed

FIG. 4. A dimerization-defective mutant of TR β . The bacterially expressed mutant TR $\beta_{415,416}$ was assayed for homo- and heterodimer formation on TRE by gel shift analysis. A ³²P-labeled TRE_{pal} oligonucleotide (0.1 ng) was used as a probe in the electrophoretic mobility shift assays. TR β (0.5 pmol), the mutant TR $\beta_{415,416}$ (0.5 pmol), and RXR α (2 pmol) were used in the assays as indicated. TR-RXR and TR-TR represent the hetero- and homodimeric receptor complexes, respectively.

FIG. 5. Dimerization-defective TR β LBD suppresses TR-mediated transcriptional silencing. Increasing amounts of the LBD peptide from 145 to 456 of the mutant TR $\beta_{415,416}$ (3.0, 5.5, and 8.0 pmol) were added to the cell-free transcription reaction mixtures in the presence or in the absence of TR β (1 pmol). The RNA transcripts generated from the test template were quantitated by densitometry and normalized with respect to the AdML transcripts as described above. The results are shown below the gel. Three independent sets of the same experiment were performed, and the results of a representative experiment are shown. The standard error of the mean values ranged between 1 and 5%.

efficiently for the silencing activity of unliganded TR β in cell-free transcription extracts, indicated that this LBD peptide bound to the corepressor with high affinity. We therefore reasoned that the LBD-corepressor complex might be stable enough in solution that one could deplete HeLa nuclear extracts of the putative corepressor activity with immobilized LBD peptide. Our approach involved the expression of the TR β LBD (positions 145 to 456) as a fusion protein with GST in *E. coli*. The GST-LBD fusion protein was immobilized on glutathione affinity resin. We then incubated HeLa nuclear extracts with resins bound to GST (control), hormone-free GST-LBD, or hormone-bound GST-LBD. After incubation, the resin was removed and the resulting extracts (supernatants) were tested for their ability to support TR-mediated silencing of a TRE-linked promoter in cell-free transcription reactions. One would predict that the nuclear extracts treated with the control GST resin (without LBD) would retain the corepressor and would therefore support efficient receptor-mediated silencing of basal RNA synthesis. In contrast, the extracts treated with immobilized GST-LBD should be either partly or fully devoid of the corepressor activity and hence would be expected to display impaired silencing by unliganded TR. Furthermore, since the LBD peptide from 145 to 456

retains the ability to bind thyroid hormone with high affinity, we could assess the effect of hormone binding on receptor-corepressor interaction by using immobilized, hormone-bound GST-LBD. It is pertinent to mention here that we preferred to use the longer LBD peptide (positions 145 to 456) rather than the shorter LBD peptide (positions 145 to 260) in this experiment because (i) the longer peptide competed for TR-mediated silencing more efficiently than the shorter peptide and (ii) the longer peptide bound to T₃ whereas the shorter peptide could not.

The results of the experiment are shown in Fig. 9. We found that, as predicted, the addition of increasing concentrations of exogenous, unliganded TR β to the GST-treated HeLa extracts led to efficient silencing of basal transcription from the target promoter (Fig. 9, lanes 2 and 3). The extracts treated with GST-LBD resin, on the other hand, exhibited either no or markedly diminished transcriptional repression by the receptor (lanes 5 and 6). More interestingly, extracts preincubated with ligand-bound GST-LBD supported silencing by unoccupied TR β as efficiently as did the GST-treated extracts. Quantitation of the results (Fig. 9) revealed that whereas TR (1 pmol) inhibited nearly 80% of basal level of RNA synthesis in extracts treated with either GST (lane 2) or T₃-bound GST-LBD (lane 9), it failed to inhibit basal transcription significantly in extracts treated with unliganded GST-LBD (lane 5). A higher dose of TR (2 pmol) led to almost 90% inhibition of basal transcription in extracts treated with either GST (lane 3) or hormone-bound GST-LBD (lane 10) but showed only a 40% inhibition in extracts treated with hormone-free GST-LBD (lane 6). The levels of RNA synthesis in lanes 8, 9, and 10, appeared to be generally elevated compared with those in lanes 1 to 7. This was due to the use of smaller amounts of internal control adenovirus major late promoter (AdML) templates in lanes 8, 9, and 10. We noted in multiple experiments that the basal levels of transcription were essentially similar in the GST-treated and the LBD-GST-treated extracts, indicating that the LBD treatment did not cause a significant depletion of any basal transcription initiation factor.

We interpret the results presented in Fig. 9 as follows. In the extracts precleared with GST, the corepressor was present and bound to the LBD of TR β to form a receptor-corepressor complex, which is the active agent mediating silencing. Removal of the limiting amount of this regulatory molecule from nuclear extracts upon incubation with immobilized, unliganded LBD peptide led to impaired silencing by TR in the depleted extracts. Hormone-bound LBD, in contrast, failed to associate with the corepressor. Preincubation with ligand-occupied, immobilized LBD therefore failed to deplete the corepressor activity from the extracts. The corepressor in these extracts remained available to generate the TR-corepressor complex and thereby promoted silencing.

DISCUSSION

Recent studies indicated that certain nuclear factors associate specifically with the members of the steroid-TR superfamily to regulate their functional activities. Cavailles et al. and Halachmi et al. have identified nuclear proteins that interact specifically with hormone-complexed, transcriptionally active estrogen receptors (14, 27). Using the yeast two-hybrid system, Lee et al. have identified several nuclear proteins termed TR-interacting proteins (Trips), that interact with both TR and RXR in a ligand-dependent fashion (33, 34). One of these proteins, Trip1, is an apparent human homolog of the yeast transcriptional mediator, Sug1 (34). The functional significance of other Trips however, remains unresolved. Fondell et

FIG. 6. Identification of a relatively small region of TR β LBD that competes for TR-mediated transcriptional silencing. (A) TR β (1 pmol) and the LBD peptide from 145 to 260 (30 pmol), were added to cell-free transcription reaction mixtures as indicated. T₃ (1 μ M) was added to the reaction mixture in lane 3. Quantitation of the transcripts generated by the test template was performed by densitometry followed by normalization with respect to the internal control AdML signals as described above. The results are shown below the gel. (B) Increasing amounts of TR β LBD peptide from 145 to 260 (5, 15, and 25 pmol) were added to the cell-free transcription reaction mixtures in the presence of TR β (1 pmol). Quantitation of the results is shown below the gel. Three independent sets of the same experiment were performed, and the results of a representative experiment are shown. The standard error of the mean values ranged between 1 and 5%.

al. observed that unliganded TR α or TR α -RXR β repressed basal transcription from a TRE-linked promoter in a transcription system consisting of purified basal transcription factors (20). The overall repression in this purified system was however, remarkably less efficient compared to that in unfractionated HeLa nuclear extracts. These workers speculated that an additional factor(s) that enhances TR-mediated repression may exist in nuclear extracts (20). Here we report that a C-terminal LBD fragment (positions 145 to 456) of TR β efficiently inhibited the silencing activity of TR in cell-free transcriptional extracts of HeLa cells. Our results suggest that this LBD peptide bound and therefore competed for a limiting amount of a soluble cofactor, termed corepressor, that plays an essential role in the receptor-mediated silencing pathway.

In vitro studies in our laboratory and elsewhere indicated that both TRs, TR α and TR β , interact directly with the initiation factor TFIIB in the absence of hormone (4, 20, 26; our unpublished observation). We therefore considered the possibility that in cell-free competition experiments, the LBD fragment reverses silencing by directly interfering with the interaction of TR β with TFIIB or other basal transcription initiation factors. Several lines of evidence, however, suggest

that this scenario is rather unlikely. First, the LBD fragment, which lacked the DNA-binding domain and therefore could not bind to the TRE in the promoter, did not interfere with basal RNA synthesis when added alone to the transcription reaction. These results indicated a lack of stable and direct functional interaction between the LBD peptide and the basal transcription machinery under cell-free transcription conditions. Second, we identified the region from 145 to 260 in the LBD as a potential corepressor-binding site. Previous in vitro protein-protein interaction studies by Baniahmad et al. established that this region does not interact with TFIIB (4). Third, treatment of HeLa extracts with immobilized, unliganded LBD, which apparently depleted corepressor activity, did not significantly affect the basal level of transcription supported by these extracts.

A significant amount of endogenous RXRs exists in HeLa nuclear extracts, and these, when combined with exogenous TR β , form exclusively heterodimers on TRE_{pal} or TRE_{DR+4} (reference 36 and our unpublished observation). It is thus likely that TR-RXR heterodimers rather than TR-TR homodimers may represent the functional form of TR in HeLa nuclear extracts. One can argue that the LBD peptide released

FIG. 7. RAR α LBD suppresses TR β -mediated transcriptional silencing. RAR α LBD peptide from positions 136 to 462 (7.5 pmol for lanes 3 and 5; 15 pmol for lanes 4 and 6) was added to the cell-free transcription reaction mixtures in the presence or absence of TR β (1 pmol). The concentration of the RAR LBD peptide was determined by comparing the intensity of the stained peptide band with that of TR β in SDS-PAGE. The plotted results represent an average of three independent experiments. The standard error of the mean values ranged between 1 and 5%.

silencing by inhibiting effective DNA binding of the repressor by sequestering TR or RXR into inactive TR-LBD homodimers or RXR-LBD heterodimers (22, 23). Our results, however, showed that (i) a high molar excess of the LBD fragment of TR β failed to compete with the TR-RXR heterodimers for DNA binding and (ii) the LBD of a mutant TR β , which is incapable of forming either homo- or heterodimers, competed efficiently for TR-mediated silencing. The inhibitory factor in HeLa extracts, which is titrated out by the LBD during reversal of silencing, is therefore clearly distinct from the receptor itself or RXR. Furthermore, these results demonstrated that heterodimerization with RXR is not a prerequisite for the LBD-corepressor association.

Our results bear a striking resemblance to a recent report by Casanova et al. (13). These workers performed transient-transfection studies to demonstrate that coexpression of an LBD peptide (positions 120 to 410) of chicken TR α and a chimeric protein containing TR α , positioned between a Gal4 DNA-binding domain and a herpes simplex virus protein 16 (VP16) activation domain, activated transcription from a Gal4-linked promoter in the absence of thyroid hormone. This transcriptional activation was reversed in the presence of T₃. These workers postulated the following scenario to explain their results. A cellular inhibitory factor bound to TR in the chimeric protein in the absence of ligand to form a receptor-inhibitor complex that suppressed gene activation. When the LBD fragment was coexpressed, it competed with the chimeric protein for the limiting amount of inhibitor, and this led to the release of transcriptional inhibition. Binding of ligand to the LBD fragment apparently dissociated the inhibitor-LBD complex. The released inhibitor then bound to the chimeric TR, which

itself did not bind to the ligand, to reconstitute gene repression.

In spite of many similarities, our results differ markedly from those of Casanova et al. with regard to the location of the site of interaction of the putative inhibitor with the LBD. Casanova et al. suggested that a conserved heptad motif containing leucines (amino acids 365 to 372) within the LBD of chicken TR α plays a critical role in binding the inhibitory factor (13). Our experiments with human TR β , in contrast, identified the region from 145 to 260 as a potential corepressor-binding site. This region is situated far upstream of the region from 416 to 423 containing the predicted ninth heptad repeat in the LBD of TR β (1). We demonstrated that mutations of the conserved leucine residue at position 416 and an adjacent lysine residue at position 415 within the conserved heptad in TR β severely impaired both TR-TR homodimerization and TR-RXR heterodimerization but did not affect the interaction of the LBD with the putative corepressor. This apparent discrepancy between our results and those of Casanova et al. is intriguing but could arise from the use of two different types of TRs, TR α and TR β , in their and our experiments, respectively.

Our results are in good agreement with those reported recently by Baniahmad et al., who demonstrated in transfection studies that two separate C-terminal regions of hTR β , positions 168 to 259 and positions 260 to 456, are necessary for gene silencing (4). The fragment from 260 to 456 has also been shown to interact with the general transcription initiation factor, TFIIB (4). However, this interaction is clearly not sufficient for silencing and needs the cooperation of the other fragment from 168 to 259 (4). In transient-transfection experiments, the TR β LBD fragment from 168 to 259 competed efficiently for transcriptional silencing activity of full-length TR β in the absence of thyroid hormone (6). In contrast, the other LBD fragment, from 260 to 456, did not compete appreciably for TR-mediated silencing under similar conditions (6). These results are consistent with our finding that the corepressor-binding site lies within the region from 145 to 260 in the LBD of TR β . We speculate that the binding of the corepressor

FIG. 8. RAR α LBD complexed with RA fails to suppress TR β -mediated transcriptional silencing. RAR α LBD peptide from 136 to 462 (10 pmol) was added to the cell-free transcription reaction mixture in the presence or in the absence of TR β (0.5 pmol) and all-*trans* retinoic acid (1 μ M) as indicated. The plotted results represent an average of three independent experiments. The standard error of the mean values ranged between 1 and 5%.

FIG. 9. Depletion of the corepressor activity from HeLa nuclear extracts (NE) impairs transcriptional silencing by TR β . Three aliquots (50 μ g each) of HeLa nuclear extracts were pretreated with immobilized GST, GST-LBD, or GST-LBD-T₃ and used in cell-free transcription reactions. TR β (0, 1, 2, or 3 pmol) was added to the reaction mixtures as indicated. The RNA transcripts generated from the test template were quantitated by densitometry and normalized with respect to the internal-control AdML transcripts. The results are shown below the gels. The value of the basal transcription for each resin-treated aliquot of nuclear extract was adjusted to 100% (lanes 1, 4, and 8). The results of a single experiment representative of three independent experiments are shown. The standard error of the mean values ranged between 1 and 5%.

to this upstream region from 145 to 260 may influence the interaction of the downstream region from 260 to 456 with the basal transcription machinery. These results thus provide an initial glimpse into a likely mechanism by which multiple C-terminal motifs of TR β may interact with a specific regulatory factor(s) and the basal transcription machinery to constitute silencing.

The corepressor-binding region, from 145 to 260, overlaps closely with the D domain of the receptor, which is commonly considered the hinge region between the DNA-binding domain and the hormone-binding region. The length and primary structure of the D domain are well conserved in all cellular TRs and in the oncogenic variant, v-erbA, indicating a potential role of this region in biological function of the receptor (15). It has indeed been reported that the sequences within this domain are critical for the oncogenic (transforming) function of v-erbA (16). The hinge domain of TR β also plays an essential role in hormone binding (38). Lee and Mahdavi reported

that point mutations within this region resulted in a loss of transcriptional regulatory activities of TR α (35). A natural mutation at amino acid 229 within the D domain of human TR β is responsible for clinical phenotypes in a family with generalized resistance to thyroid hormone (8). Our present observation that this region interacts with an inhibitory factor in the absence of ligand to repress transcription provides a novel clue to the role of this receptor domain in gene regulatory mechanisms.

Hormone binding to TR triggers a conformational change in the receptor molecule (37, 44). Hormone binding to the receptor also reverses its silencing function. We provide strong biochemical evidence suggesting that the hormonal ligand influences the interaction between the receptor and the corepressor and thereby modulates the repressor function. We noted that unliganded RAR LBD competed for silencing by TR β , presumably by interacting with the limiting, common corepressor. Hormone-bound RAR LBD, in contrast, failed to

FIG. 10. Outlines of a hypothetical model for transcriptional regulation by TR. (A) In the absence of TR, the RNA polymerase II transcription machinery may direct a low basal level of mRNA synthesis from a TRE-linked promoter. IIA, IIB, IID, IIE, IIF, IIH, and Pol II denote the transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIIF, TFIIF, and RNA polymerase II, respectively. (B) A TR-RXR heterodimer occupying the TRE blocks basal RNA synthesis in the absence of thyroid hormone, by either interfering with the assembly or impairing the function of a preinitiation complex at the TATA box. A corepressor, interacting with the LBD of TR, critically modulates the interaction between TR and the basal transcription machinery. (C) In the presence of excess LBD fragment, the inhibitory corepressor is titrated away and the TR-mediated transcriptional block is released. (D) Binding of hormonal ligand to TR alters its conformation and disrupts the receptor-corepressor complex. Consequently, activation domains are exposed in the ligand-bound receptor and interact with the basal transcription machinery either directly or via a mediator (such as a coactivator) to bring about transactivation.

compete for TR-mediated silencing. These results suggested strongly that ligand binding to RAR LBD disrupted its interaction with the corepressor. This view was reinforced by our experiments involving protein affinity methods to deplete the

corepressor activity from HeLa nuclear extracts. We observed that unliganded, immobilized GST-LBD stably interacted with the corepressor through protein-protein interactions and removed it from HeLa extracts. The ligand-bound LBD, how-

ever, failed to deplete nuclear extracts of the corepressor activity, apparently because of the failure to interact with this regulatory molecule. These findings shed light on the functional role of the hormonal ligand during the reversal of TR-mediated gene repression.

Previous studies in our laboratory suggested that unliganded TR β negatively modulates basal transcription by directly influencing one or more steps leading to the formation of a functional preinitiation complex (45). The assembly of an initiation complex is a highly ordered process that proceeds through sequential recruitment of RNA polymerase II and other general transcription factors to the core promoter (10, 11, 18, 47). *In vitro* studies in our laboratory with highly purified protein preparations indicated that hormone-free TR β or TR β -RXR heterodimer can interact with the TFIIB-TATA-binding protein (TBP)-TATA complex, which is an early intermediate during initiation complex assembly (45). We also demonstrated that the binding of TR β to the TFIIB-TBP-TATA complex was significantly reduced in the presence of T $_3$. These results led us to propose that transcriptional silencing by TR may involve interactions between unliganded receptor and core promoter complexes such as TFIIB-TBP-TATA, resulting in the formation of a transcriptionally inactive complex. Our present findings add another layer of complexity to this gene regulatory model (Fig. 10). We now propose that an additional regulatory molecule, a corepressor, is necessary to stabilize the inhibitory interactions between the unliganded receptor and the basal transcription machinery that lead to the arrest of transcription complex assembly (Fig. 10B). These interactions, critically modulated by the corepressor, may freeze the early intermediates in a nonfunctional conformation unable to proceed through functional preinitiation complex assembly. We postulate also that the ligand-induced release of the corepressor reduces or eliminates the abortive interactions between the receptor and the initiation factors, thereby allowing the assembly of productive transcription initiation complexes at the target promoter (Fig. 10D). In addition, activation domains that had been hidden in the unliganded receptor may become unmasked and available for interaction with a coactivator(s) and/or basal transcriptional apparatus.

The molecular interactions between the receptor, the corepressor, and the components of the basal transcription machinery may occur in a number of possible ways. The corepressor may function as a physical link between the promoter-bound receptor and the basal transcription machinery, making contacts with both. TR may also make direct contacts with the basal transcription machinery, independent of the corepressor. It has been reported that TR β is able to repress basal RNA synthesis in a purified, reconstituted transcription system, although the magnitude of this repression was much weaker than that in an unfractionated system (20). We observed also (Fig. 9, lane 6) that upon addition of high concentrations of the hormone-free TR β , appreciable suppression of basal transcription occurred in a corepressor-depleted extract. These results indicate that in cell-free transcription systems, TR may undergo direct but weak inhibitory interactions with the basal transcription machinery in the absence of the corepressor. We propose that the role of the corepressor is to promote repression by stabilizing these interactions between the repressor and the basal factors. This concept of corepressor function is a virtual mirror image of the proposed mechanism of action of coactivators, which are thought to be adaptor or mediator molecules that couple transcriptional activators to basic transcription machinery during activation processes (41). A number of such coactivators have been isolated and characterized recently (14, 24, 28, 32). The functional evidence presented

here points to the existence of a corepressor for TR in HeLa nuclear extracts and opens the way to its biochemical isolation. Further studies with the cell-free gene expression system are required to clarify the nature of this corepressor activity and to decipher its mechanism of action.

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ADDENDUM

After this paper was submitted for review, three papers reported the isolation of two distinct but structurally related corepressor molecules, N-CoR and SMRT, which associate with unliganded TR or RAR in a ligand-reversible manner (14a, 29a, 31a).

REFERENCES

1. Au-Fliegner, M., E. Helmer, J. Casanova, B. Raaka, and H. H. Samuels. 1993. The conserved ninth C-terminal heptad in thyroid hormone and retinoic acid receptors mediates diverse responses by affecting heterodimer but not homodimer formation. *Mol. Cell. Biol.* **13**:5725-5737.
2. Bagchi, M. K., S. Y. Tsai, and M.-J. Tsai. 1995. *In vitro* reconstitution of progesterone-dependent RNA transcription in nuclear extracts of human breast carcinoma cells. *In vitro* transcription and translation protocols. *Methods Mol. Biol.* **37**:107-121.
3. Bagchi, M. K., S. Y. Tsai, N. L. Weigel, M.-J. Tsai, and B. W. O'Malley. 1990. Regulation of *in vitro* transcription by progesterone receptor: characterization and kinetic studies. *J. Biol. Chem.* **265**:5129-5134.
4. Baniahmad, A., I. Ha, D. Reinberg, S. Y. Tsai, M.-J. Tsai, and B. W. O'Malley. 1993. Interaction of human thyroid hormone receptor β with transcription factor TFIIB may mediate target gene derepression and activation by thyroid hormone. *Proc. Natl. Acad. Sci. USA* **90**:8832-8836.
5. Baniahmad, A., A. C. Kohne, and R. Renkawitz. 1992. A transferable silencing domain is present in the thyroid hormone receptor, in the v-erbA oncogene product and in the retinoic acid receptor. *EMBO J.* **11**:1015-1023.
6. Baniahmad, A., X. Leng, T. P. Burris, S. Y. Tsai, and M.-J. Tsai, and B. W. O'Malley. 1995. The τ 4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. *Mol. Cell. Biol.* **15**:76-86.
7. Baniahmad, A., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai. 1992. Kindred S thyroid hormone receptor is an active and constitutive silencer and a repressor for thyroid hormone and retinoic acid responses. *Proc. Natl. Acad. Sci. USA* **89**:10633-10637.
8. Behr, M., and U. Loos. 1992. A point mutation (Ala229 to Thr) in the hinge domain of the c-erbA β thyroid hormone receptor gene in a family with generalized thyroid hormone resistance. *Mol. Endocrinol.* **6**:1119-1126.
9. Brent, G. A., M. K. Dunn, J. W. I. Harney, T. Gulick, P. R. Larsen, and D. D. Moore. 1989. Thyroid hormone aporeceptor represses T3 inducible promoters and blocks activity of the retinoic acid receptor. *New Biol.* **1**:329-336.
10. Buratowski, S. 1994. The basics of basal transcription by RNA polymerase II. *Cell* **77**:1-3.
11. Buratowski, S., S. Hahn, L. Guarente, and P. A. Sharp. 1989. Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* **56**:549-561.
12. Burnside, J., D. S. Darling, and W. W. Chin. 1990. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. *J. Biol. Chem.* **265**:2500-2504.
13. Casanova, J., E. Helmer, S. Selmi-Ruby, J.-S. Qi, M. Au-Fliegner, V. Desai-Yajnik, N. Koudinova, F. Yarm, B. M. Raaka, and H. H. Samuels. 1994. Functional evidence for ligand-dependent dissociation of thyroid hormone and retinoic acid receptors from an inhibitory cellular factor. *Mol. Cell. Biol.* **14**:5756-5765.
14. Cavailles, V., S. Dauvois, F. L'Horset, G. Lopez, S. Hoare, P. J. Kushner, and M. G. Parker. 1995. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. *EMBO J.* **14**:3741-3751.
- 14a. Chen, J. D., and R. M. Evans. 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. *Nature (London)* **377**:454-457.
15. Chin, W. W. 1991. Nuclear thyroid hormone receptors, p. 79-96. *In* M. G. Parker (ed.), *Nuclear hormone receptors*. Academic Press, Inc., San Diego, Calif.
16. Damm, K., H. Beug, T. Graf, and B. Vennestrom. 1987. A single point mutation in erbA restores the erythroid transforming potential of a mutant avian erythroblastosis virus (AEV) defective in both erbA and erbB oncogenes. *EMBO J.* **6**:375-382.

17. Damm, K., C. C. Thompson, and R. M. Evans. 1989. Protein encoded by v-erbA functions as a thyroid hormone receptor antagonist. *Nature (London)* **339**:593–597.
18. Drapkin, R., A. Merino, and D. Reinberg. 1993. Advances in regulation of transcription by RNA polymerase II. *Curr. Opin. Cell Biol.* **5**:469–476.
19. Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**:889–895.
20. Fondell, J. D., A. L. Roy, and R. G. Roeder. 1993. Unliganded thyroid hormone receptor inhibits formation of a functional initiation complex: implications for active repression. *Genes Dev.* **7**:1400–1410.
21. Forman, B. M., J. Casanova, B. M. Raaka, J. Ghysdael, and H. H. Samuels. 1992. Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers. *Mol. Endocrinol.* **6**:429–442.
22. Forman, B. M., and H. H. Samuels. 1990. Interactions among a subfamily of nuclear hormone receptors: the regulatory zipper model. *Mol. Endocrinol.* **4**:1293–1301.
23. Forman, B. M., C. Yang, M. Au, J. Casanova, J. Ghysdael, and H. H. Samuels. 1989. A domain containing leucine-zipper-like motifs mediates novel in vivo interactions between the thyroid hormone and retinoic acid receptors. *Mol. Endocrinol.* **3**:1610–1626.
24. Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon, and R. Tjian. 1993. Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* **75**:519–530.
25. Graupner, G., K. N. Wills, M. Tzukerman, X.-K. Zhang, and M. Pfahl. 1989. Dual regulatory role for thyroid hormone receptors allows control of retinoic acid receptor activity. *Nature (London)* **340**:653–656.
26. Hadzic, E., V. Desai-Vajnik, E. Helmer, S. Guo, S. Wu, N. Koudinova, J. Casanova, B. M. Raaka, and H. H. Samuels. 1995. A 10-amino-acid sequence in the N-terminal A/B domain of thyroid hormone receptor α is essential for transcriptional activation and interaction with the general transcription factor TFIIB. *Mol. Cell. Biol.* **15**:4507–4517.
27. Halachmi, S., E. Marden, G. Martin, H. MacKay, C. Abbondanza, and M. Brown. 1994. Estrogen receptor-associated proteins: possible mediators of hormone-induced transcription. *Science* **264**:1455–1458.
28. Hoey, T., R. O. Weinzierl, G. Gill, J. L. Chen, B. D. Dynlacht, and R. Tjian. 1993. Molecular cloning and functional analysis of Drosophila TAF110 reveal properties expected of coactivators. *Cell* **72**:247–260.
29. Hoffmann, A., and R. G. Roeder. 1991. Purification of His-tagged proteins in non-denaturing conditions suggests a convenient method for protein interaction studies. *Nucleic Acids Res.* **19**:6337–6338.
- 29a. Horlein, A. J., A. M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, C. K. Glass, and M. G. Rosenfeld. 1995. Ligand-independent repression by the thyroid hormone repressor mediated by a nuclear receptor co-repressor. *Nature (London)* **377**:397–404.
30. Kliever, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans. 1992. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature (London)* **355**:446–449.
31. Koenig, R. J., R. L. Warne, G. A. Brent, J. W. Harne, P. R. Larsen, and D. D. Moore. 1988. Isolation of a cDNA clone encoding a biologically active thyroid hormone receptor. *Proc. Natl. Acad. Sci. USA* **85**:5031–5035.
- 31a. Kurokawa, R., M. Söderström, A. Horlein, S. Halachmi, M. Brown, M. G. Rosenfeld, and C. K. Glass. 1995. Polarity-specific activities of retinoic acid receptors determined by a co-repressor. *Nature (London)* **377**:451–454.
32. Kwok, R. P. S., J. R. Lundblad, J. C. Chrivia, J. P. Richards, H. P. Bachinger, R. G. Brennan, S. G. E. Roberts, M. R. Green, and R. H. Goodman. 1994. Nuclear protein CBP is a coactivator for the transcription factor CREB. *Nature (London)* **370**:223–226.
33. Lee, J. W., H.-S. Choi, J. Gyuris, R. Brent, and D. D. Moore. 1995. Two classes of proteins dependent on either the presence or absence of thyroid hormone for interaction with the thyroid hormone receptor. *Mol. Endocrinol.* **9**:243–254.
34. Lee, J. W., F. Ryan, J. C. Swaffield, S. A. Johnston, and D. D. Moore. 1995. Interaction of thyroid hormone receptor with a conserved transcriptional mediator. *Nature (London)* **374**:91–94.
35. Lee, Y., and V. Mahdavi. 1993. The D domain of the thyroid hormone receptor α 1 specifies positive and negative transcriptional regulation functions. *J. Biol. Chem.* **268**:2021–2028.
36. Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J. Y. Chen, A. Staub, J. M. Garnier, S. Mader, and P. Chambon. 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68**:377–395.
37. Leng, X. H., S. Y. Tsai, B. W. O'Malley, and M.-J. Tsai. 1993. Ligand-dependent conformational changes in thyroid hormone and retinoic acid receptors are potentially enhanced by heterodimerization with retinoic X receptor. *J. Steroid Biochem. Mol. Biol.* **46**:643–661.
38. Lin, K. H., C. Parkinson, and S. Y. Cheng. 1991. An essential role of domain D in the hormone binding activity of human β 1 thyroid hormone nuclear receptor. *Mol. Endocrinol.* **5**:485–492.
39. Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans. 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. *Nature (London)* **345**:224–229.
40. Murray, M. B., and H. C. Towle. 1989. Identification of nuclear factors that enhance binding of thyroid hormone receptor to a thyroid hormone response element. *Mol. Endocrinol.* **3**:1434–1442.
41. Pugh, B. F., and R. Tjian. 1990. Mechanism of transcriptional activation by Sp1: evidence for coactivators. *Cell* **61**:1187–1197.
42. Sap, J., A. Munoz, J. Schmitt, H. Stunnenberg, and B. Vennstrom. 1989. Repression of transcription mediated at a thyroid hormone response element by the v-erbA oncogene product. *Nature (London)* **340**:242–244.
43. Thompson, C. C., and R. M. Evans. 1989. Transactivation by thyroid hormone receptors: functional parallels with steroid hormone receptors. *Proc. Natl. Acad. Sci. USA* **86**:3494–3498.
44. Toney, J. H., L. Wu, A. E. Summerfield, G. Sanyal, B. M. Forman, J. Zhu, and H. H. Samuels. 1993. Conformational changes in chicken thyroid hormone receptor α 1 induced by binding to ligand or to DNA. *Biochemistry* **32**:2–6.
45. Tong, G.-X., M. R. Tanen, and M. K. Bagchi. 1995. Ligand modulates the interaction of thyroid hormone receptor β with the basal transcription machinery. *J. Biol. Chem.* **270**:10601–10611.
46. Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kim, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld. 1991. RXR β , a coregulator that enhances binding of retinoic acid, thyroid hormone and vitamin D receptors to their cognate response elements. *Cell* **67**:1251–1266.
47. Zewel, L., and D. Reinberg. 1992. Advances in RNA polymerase II transcription. *Curr. Opin. Cell Biol.* **4**:488–495.
48. Zhang, X.-K., B. Hoffmann, P. B. Tran, G. Graupner, and M. Pfahl. 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (London)* **355**:441–446.