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Activation and Repression by Nuclear Hormone Receptors: Hormone Modulates an Equilibrium between Active and Repressive States

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Transactivation-defective retinoid X and thyroid hormone receptors have been used to examine mechanisms of hormonal activation. Activation and repression of transcription by retinoid X and thyroid hormone receptors are shown to be mediated by physically distinct and functionally independent regions of the hormone binding domain. Nevertheless, the ability of receptors to respond to hormone requires communication between both functional domains. Deletion of the hormone-dependent transactivation function of the retinoid X receptor, the common subunit of heterodimeric nuclear receptors, significantly impairs hormone-dependent transcription by retinoic acid, thyroid hormone, and vitamin D receptors. The results indicate that receptors do not exist in static off and on conformations but that hormone alters an equilibrium between inactive and active states.

Members of the steroid hormone receptor and thyroid hormone receptor (TR) superfamily function as hormone-dependent transcription factors to regulate the expression of complex gene networks involved in vertebrate development, differentiation, and homeostasis. The TR subclass of the superfamily includes receptors for thyroid hormone, vitamins A and D, and prostanoids and numerous orphan receptors. Receptors in this subclass, unlike the steroid hormone receptors, appear to be constitutively nuclear and bound to DNA. Thus, hormone acts as a direct switch modulating the activity of receptors bound to DNA (for reviews, see references 22, 30, and 31).

A short carboxyl-terminal activation domain (called τc or AF-2) identified in several members of the TR subclass has been shown to be required for hormone-dependent transcription (3, 4, 11, 13, 27, 37, 45). The mechanism by which hormone modulates the activity of the τc domain and any other potential hormone-dependent transactivation functions (3, 20, 39) is not clear. Binding of hormone appears to induce a conformational change in receptors that leads to activation of transcription. Recent structural analysis of the ligand binding domains of the retinoid X receptor (RXR), retinoic acid receptor (RAR), and TR suggests that hormone binding results in the folding back of the τc domain toward the ligand binding domain (LBD) core. The loose packing of the τc domain on the surface of the LBD is thought to lead to the reorganization or stabilization of the τc domain, creating the proper surface for interaction with components of the basal transcription machinery or coactivators (5, 35, 43). Many members of the TR subclass function as obligate heterodimers, with RXR as the common heterodimeric partner (for a review, see reference 30). The requirement for the RXR τc domain in transactivation when its heterodimeric partner is activated by hormone, however, remains unclear.

Several members of the TR subclass, most notably TR and RAR, have been shown to function as repressors of transcription in the absence of hormone (2, 10, 44). Recent evidence suggests that repression is mediated by the ability of these receptors to recruit a *trans*-acting repressor (6–8, 21, 23). Direct interaction with components of the basal transcription machinery has also been implicated in repression (1, 14, 15, 40). Binding of hormone leads to both release of the *trans*-acting repressor and positive transactivation. Thus, not only does binding of hormone change receptors from inactive to active, but also in some cases hormone reverses receptor-mediated repression of transcription. Interestingly, although members of the TR subclass have been shown to positively activate transcription in the budding yeast *Saccharomyces cerevisiae*, repression by hormone receptors has not been observed in this organism (25, 33). Unlike positive transactivation, it appears that the machinery for repression of transcription is not well conserved among different species.

In this work, analysis of RXR and TR indicates that transcriptional activation and repression can be physically dissected from each other. The ability of hormone to change the functional state of receptors from repressors to activators, however, requires communication between these two functional domains. Furthermore, deletion of the τc domain of RXR leads to a dramatic decrease in transactivation by RXR-receptor heterodimers, suggesting that both members of the heterodimer participate in hormonal signaling. These results suggest that receptors are not static but exist in equilibrium, shifting between inactive-repressive and active states. Evidence that the role of hormone is to drive the equilibrium toward the active state is presented.

MATERIALS AND METHODS

Plasmids. Receptor LBD fusions were cloned by PCR amplification of human RXRα (amino acids 197 to 462 and 197 to 443) and human TRα (amino acids 121 to 410 and 121 to 390). Stop codons were included after positions 443 (RXR) and 390 (TR) for construction of the τc deletions. Amplified products were cloned into pCMXG4epi (37). The chimeric GAL4 fusions RXT and TRX were constructed by PCR amplification of human RXRα (amino acids 197 to 443) and human TRα (amino acids 121 to 390). The amplification products were inserted into *Nco*I-digested pRS305CHY-TR391-410 (RXT) and pRS305CYH-RXR444-462 (TRX) (37). The chimeric receptors were then subcloned into pCMXG4epi. At the junction between RXR and TR sequences two additional amino acids

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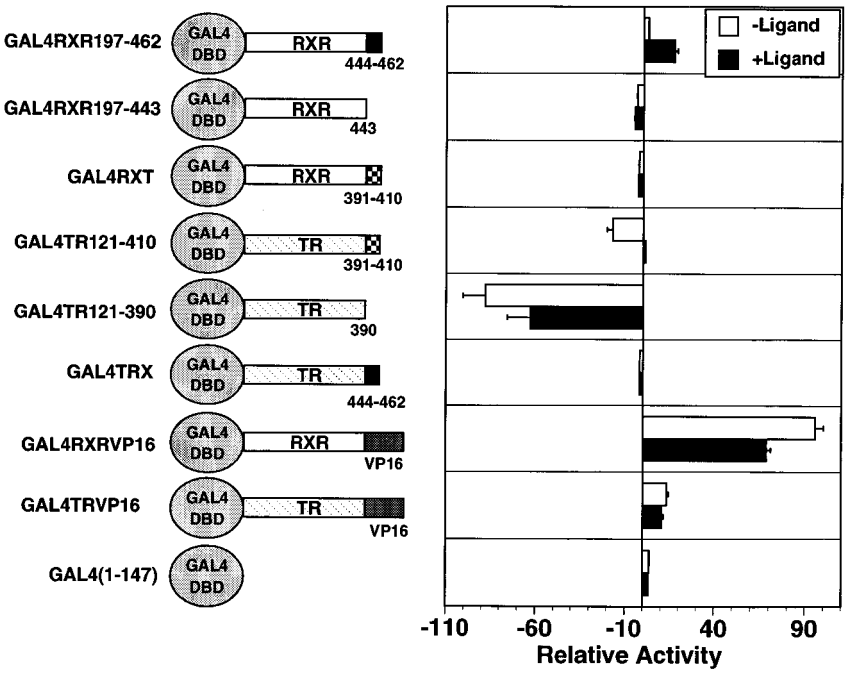

FIG. 1. Receptor activity can be modulated by combining unique repression and activation domains. Fusions between the GAL4 DNA binding domain (GAL4 DBD) and RXR (amino acids 197 to 462 and 197 to 443), TR (amino acids 121 to 410 and 121 to 390), and the chimeras RXT, TRX, TRVP16, and RXRVP16 (see Materials and Methods) were transfected into CV1 cells along with the reporter GAL3-TK-LUC. After transfection, CV1 cells were cultured in the presence or absence of 1 μM 9-*cis* retinoic acid (RXR) or 1 μM T_3 (TR) for 36 h. The basal activity observed with the reporter alone (approximately 10 relative light units) was set at 1. Activity relative to that with the reporter alone is presented. Transfection results were normalized by cotransfection with a β-galactosidase expression plasmid (see Materials and Methods). Open boxes, RXR197-443; black boxes, the RXR τc domain (amino acids 444 to 462); stippled boxes, TR121-390; checkered boxes, the TR τc domain (amino acids 391 to 410); gray boxes, VP16 activation domain (amino acids 412 to 490).

(alanine-methionine) are added. The chimeric GAL4 fusions RXRVP16 and TRVP16 were constructed by PCR amplification of human RXRα (amino acids 197 to 443), human TRα (amino acids 121 to 390), and VP16 (amino acids 412 to 490). The amplified receptor and VP16 fragments were cloned directly into pCMX-GAL4-N (provided by K. Umesono). Expression constructs for full-length human RXRα, human RARα, and human TRβ; luciferase reporter constructs with two copies of synthetic direct repeats (AGGTCA) spaced by 3, 4, and 5 nucleotides; and the reporter construct GAL3-TK-Luc containing three binding sites for GAL4 have been described previously (37, 41). RXR1-443 was amplified by PCR and cloned into expression plasmid pCMX (41). For expression of GAL4-RXR fusions in *S. cerevisiae*, amino acids 225 to 462 and 225 to 443 of human RXRα were amplified by PCR and cloned into vector pG6H (37). A stop codon was included after position 443 for construction of the τc deletion.

Transfection. Transfection of CV1 cells was carried out as described by Schulman et al. (37). Cells were incubated for 36 h at 37°C with or without 9-*cis* retinoic acid, T_3 (3,3′,5-triiodo-L-thyronine), or vitamin D_3. The RAR-specific ligand AM580 [4-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthamido)benzoic acid] was also used. Cells were harvested after 36 h of growth at 37°C. Luciferase activity of each sample was normalized by the level of β-galactosidase activity.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays were carried out as described by Chen and Evans (7). The probe was an oligonucleotide generated from the retinoic acid response element present in the promoter of the mouse RARβ gene (38).

Yeast strains and methods. The strain Y190 (*MAT***a** *gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,112 cyh*^{r} *URA3*::*GAL1*→*lacZ LYS2*::*GAL1*→*HIS3*; gift of S. Elledge, Baylor College of Medicine) was used for all experiments. Transformation was carried out as described elsewhere (12). β-Galactosidase assays were carried as described by Schulman et al. (37). A minimum of three independent transformants were assayed.

RESULTS

The τc domain is required for hormone-dependent relief of repression. We and others have recently shown that a small amphipathic α-helix at the carboxyl terminus of RXR and TR, called the τc domain or AF-2, is necessary for hormone-dependent transcription (3, 4, 11, 13, 27, 36, 37, 45). Deletion of the τc domain from GAL4-RXR and GAL4-TR LBD fusions not only eliminates hormone-dependent transcription but also reduces the level of transcription observed in the absence of hormone five- to sevenfold below that observed with the intact GAL4 fusions or the GAL reporter alone (Fig. 1). Thus, removal of the τc domain produces receptors that reduce or repress hormone-independent transcription better than the natural receptors. Similar results have been observed for τc domain deletions of full-length RXR, TR, and RAR (3, 9, 27, 36a). The level of repression observed with natural and truncated receptors correlates with their relative affinities for members of the recently identified family of TR- and RAR-associated corepressors (TRACs) (7, 8, 21, 23).

When fused to heterologous DNA binding domains, the τc domains of RXR and TR activate transcription constitutively (3, 4, 11, 13, 27, 37). Nevertheless, transactivation by the intact receptors requires hormone (Fig. 1). If repression and activation are mediated by two independent functional domains, the role of hormone may be to relieve or reduce repression, allowing the positive effect of the constitutively active τc domain to dominate. To test the ability of hormone to relieve repression in the absence of activation, the effect of hormone on the activity of the RXR τc deletion (GAL4RXR197-443) was examined. This truncated receptor binds 9-*cis* retinoic acid with an affinity similar to that of the intact receptor (27, 37). As shown in Fig. 1, addition of 9-*cis* retinoic acid has no effect on the level of transcription by GAL4RXR197-443. Therefore, although activation and repression functions can be physically separated, both functional domains are required to respond to hormone. Similar results are observed for TR and RAR (Fig. 1 and data not shown).

The RXR τc domain is required for transactivation by heterodimers. Identifying an activity in RXR that reduces or re-

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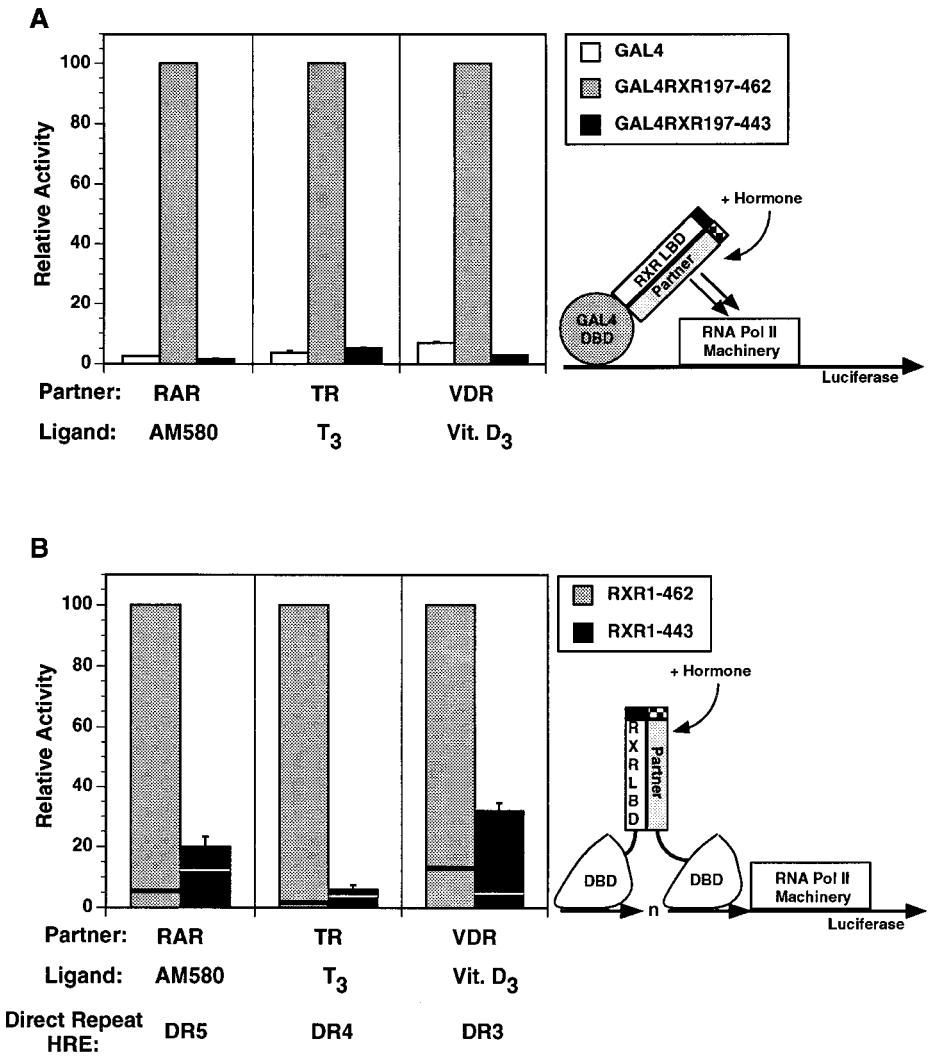

FIG. 2. Deletion of the RXR τc domain reduces transactivation by RAR, TR, and VDR heterodimers. (A) Constructs expressing the LBDs of human RARα, human TRβ, human VDR, and a reporter with three GAL4 binding sites (GAL3-TK-LUC) were transfected into CV1 cells along with the GAL4 DNA binding domain (amino acids 1 to 147) or with fusions between the GAL4 DNA binding domain and the RXR LBD (amino acids 197 to 462) or the τc domain deletion (amino acids 197 to 443). After transfection, CV1 cells were cultured in the presence or absence of 100 nM AM580 (RAR), T_3 (TR), or vitamin D_3 (VDR) for 36 h. Activity observed in the presence of the RXR LBD fusion (GAL4RXR197-462) was set at 100%. DBD, DNA binding domain; Pol II, polymerase II. (B) Constructs expressing full-length human RARα, human TRβ, or human VDR were cotransfected into CV1 cells along with constructs expressing full-length human RXRα (RXR1-462) or the RXR τc domain deletion (RXR1-443). Luciferase reporters containing two copies of synthetic direct repeats spaced by 5 (RAR), 4 (TR), or 3 (VDR) bp were also included in the transfections. Cells were treated as described for panel A. Activity observed in the presence of RXR1-462 was set at 100%. The lines in the bars indicate the relative activity in the absence of hormone. Transfection results in both panels were normalized by cotransfection with a β-galactosidase expression plasmid (see Materials and Methods).

presses transcription was unexpected. This activity, if present in an intact receptor, could affect several independent hormonal signaling pathways through the ability of RXR to form heterodimers with multiple receptors (for reviews, see references 24 and 30). To examine the ability of RXR to negatively affect other signaling pathways, transfection experiments were carried out to compare the activity of RXR-receptor heterodimers using either intact RXR or the RXR τc domain deletion. As shown in Fig. 2, removal of the τc domain from RXR significantly reduces transactivation by RXR-RAR, -TR, and -vitamin D_3 receptor (VDR) heterodimers either in transfection assays using a GAL4-based system (Fig. 2A) (16) or when full-length receptors are assayed on the appropriate hormone response elements (Fig. 2B) (41). The experiments in Fig. 2 were carried out in the presence of hormone specific for the RXR heterodimeric partner, indicating that the RXR τc domain is necessary for hormone-dependent transactivation by heterodimers.

Repression of transcription by TR and RAR has been shown to be mediated by a recently identified family of corepressors (TRACs). Members of this family bind to receptors in the absence of hormone and are released from receptors in the presence of hormone (7, 8, 21, 23). Interestingly, deletion of the τc domain of RAR produces a receptor that binds hormone but does release corepressors. This mutant RAR, like the RXR τc domain deletion, is a constitutive repressor (7, 21). To determine if removal of the τc domain of RXR also affected interaction or release of corepressors, the electrophoretic mobility shift experiment shown in Fig. 3 was carried out. Incubation of RXR-RAR heterodimers with the silencing mediator of retinoid and thyroid hormone receptors (SMRT) (7) produces an RXR-RAR-SMRT complex detectable as a super-

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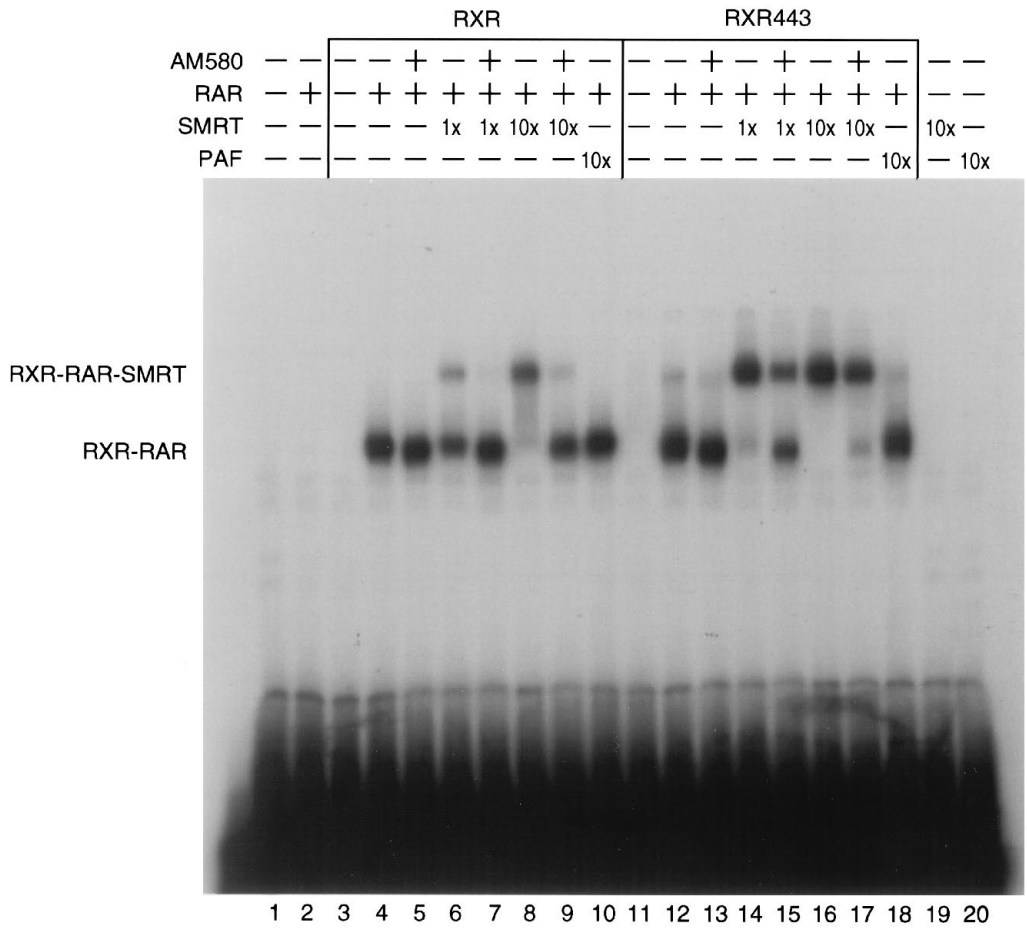

FIG. 3. Deletion of the RXR τc domain increases the interaction between RXR-RAR heterodimers and the corepressor SMRT. Interaction between RXR-RAR heterodimers and SMRT was examined by electrophoretic mobility shift assay using a βRE response element as described in Materials and Methods. Heterodimers were formed with full-length RXR (lanes 3 to 10) or RXR443 (τc domain deletion) (lanes 11 to 18) transcribed and translated in vitro. Samples in lanes 5 to 9, 14 to 17, and 19 contain either 60 ng (1×) or 600 ng (10×) of recombinant GST-SMRT. Samples in lanes 10, 18, and 20 contain 600 ng of GST-PAF. PAF is a PML-associated factor and serves as a negative control. Lanes 5, 7, 9, 13, 15, and 17 contain the RAR-specific ligand AM580 (100 nM).

shift (lanes 6 and 8). Addition of the RAR-specific ligand AM580 releases greater than 90% of the SMRT from the complex (lanes 7 and 9). When a heterodimer is formed using the RXR τc domain deletion (RXR443) (lanes 11 to 18), addition of SMRT produces an increased supershift compared with the full-length RXR (compare lanes 14 and 16 with lanes 6 and 8), indicating tighter binding of SMRT. Addition of AM580 to the RXR443-RAR-SMRT complex leads to only a partial release of SMRT (compare lanes 15 and 17 with 7 and 9). Identical results are observed with RXR443-TR and RXR443-VDR heterodimers (data not shown). The results in Fig. 3 indicate that the τc domain of RXR can significantly affect the binding of SMRT to heterodimers and is required for the complete release of corepressors when the partner is activated by hormone.

Receptor activity can be modulated by combining unique repression and activation domains. The results in Fig. 1 show that when the τc domains of RXR and TR are removed, the repressing domain of TR (GALTR121-390) is significantly stronger than the repressing domain of RXR (GALRXR197-443). Interestingly, we have previously shown that when fused to GAL4, the τc domain of RXR is a four- to fivefold stronger positive activator than the TR τc domain (37). This relative difference in the activities of the isolated functional domains suggests that the level of transcription is determined by the ability of activation and repression to counteract or balance each other. For instance, TR combines a relatively strong repressor with a relatively weak activator. This combination produces a receptor that represses transcription in the absence of hormone. RXR, on the other hand, represents a combination of a relatively weak repressor with a relatively strong activator, producing a receptor that is essentially neutral. If such a counterbalancing hypothesis is correct, then the properties of chimeric GAL4 fusions in which the RXR and TR τc domains are interchanged or replaced can be predicted. As shown in Fig. 1, the chimera GAL4RXT, in which the relatively weak τc domain of TR is substituted for the relatively strong τc domain of RXR, reduces transcription fourfold compared with the intact RXR fusion (GAL4RXR197-462). Conversely, the second chimera, GAL4TRX, combining the relatively strong repression of TR with the relatively strong τc domain of RXR, is a significantly weaker repressor compared with the intact TR fusion (GAL4TR121-410). Replacement of the τc domains of RXR and TR with the strong activation domain of VP16 produces constitutively active fusions (GAL4TRVP16 and GAL4RXR VP16 [Fig. 1]). As expected, however, the GAL4RXRVP16 is a stronger activator than GAL4TRVP16.

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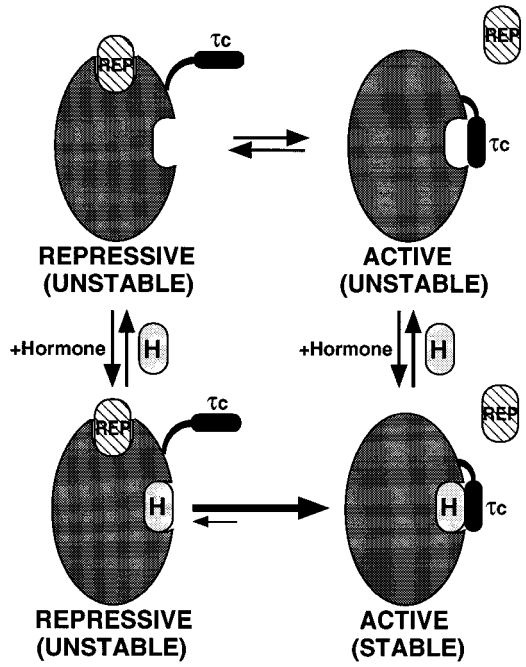

FIG. 4. An equilibrium model for hormone-dependent transactivation. (Top) In the absence of hormone, receptors exist in an equilibrium between repressive and active states. When in the repressive state (left), the receptor is in a conformation permissive for binding a *trans*-acting repressor (REP). In the active state (right), the receptor assumes a conformation in which the τc domain is structured or stabilized and the repressor is unable to bind. The position of the equilibrium is determined by the relative strengths of the repression and activation domains as described in the text. (Bottom) The presence of hormone (H) drives the equilibrium to the active state by destabilizing the repressive state and/or stabilizing the active state.

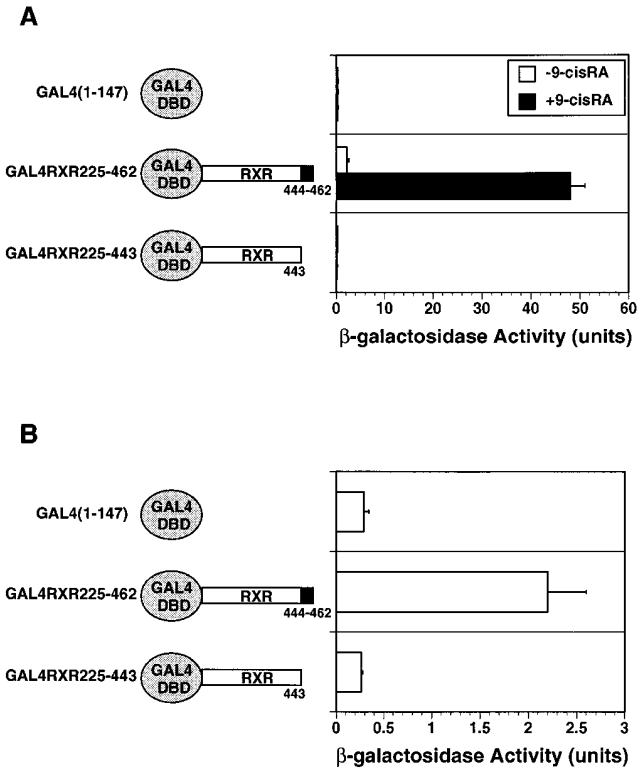

FIG. 5. Hormone-independent transactivation can be detected in *S. cerevisiae*. High-copy-number plasmids expressing the GAL4 DNA binding domain (DBD) (amino acids 1 to 147) or fusions between the GAL4 DNA binding domain and human RXRα (amino acids 225 to 462 and 225 to 443) were transformed into *S. cerevisiae* Y190 containing an integrated GAL1-LacZ reporter (see Materials and Methods). (A) Hormone-dependent transcription. *S. cerevisiae* cells bearing each plasmid were cultured and assayed in the presence or absence of 1 μM 9-*cis* retinoic acid as described in Materials and Methods. (B) Hormone-independent transcription. Enlarged version of the hormone-independent activity shown in panel A.

An equilibrium model for hormone-dependent transactivation. The ability of τc domains to counterbalance repression in the absence of hormone appears paradoxical. Transactivation by RXR and TR is hormone dependent. Nevertheless, in order to counterbalance repression, the τc domain must be activating transcription in the absence of hormone. This apparent paradox can be resolved by a model in which in the absence of hormone, receptors exist in equilibrium, flip-flopping between repressive and active states (Fig. 4). These two states could represent a conformation that can bind *trans*-acting repressors and has a disordered or inactive τc domain (repressive state) (Figure 4, top left) and a conformation that cannot bind *trans*-acting repressors and has an ordered or active τc domain available for interaction with positively acting factors (active state) (Fig. 4, top right). The length of time that an individual receptor spends in each state would be determined by the affinity of the repressing and τc domains for the appropriate *trans*-acting factors and/or by the stability of each conformation (Fig. 4, top). The role of hormone binding in this model is to drive the equilibrium to the active state (Fig. 4, bottom). The observation that the τc domain is required for both hormone-dependent relief of repression and hormone-induced transcription (Fig. 1) suggests that the τc domain participates in the change from repressive to active states directly, perhaps by competing with corepressors for binding to the LBD. Alternatively, the requirement for the τc domain may be indirect, for instance, if the τc domain is required to initiate or maintain a conformational change that leads to loss of repressor binding. Altering the relative activity of each domain changes the equilibrium to produce receptors with different levels of activity, as illustrated by the chimeras in Fig. 3.

If, as suggested by the equilibrium model proposed above, unliganded receptors are shifting between repressive and active states, it should be possible to detect activation in the absence of hormone. This hormone-independent activation, however, should remain dependent on the τc domain. To test this prediction, we took advantage of the ability to express GAL4-LBD fusions in *S. cerevisiae*. Previous work has indicated that receptors respond normally to hormone when expressed in *S. cerevisiae* (17, 19, 25, 28, 29). The absence of *trans*-acting factors required for repression by hormone receptors in *S. cerevisiae*, however, allows one to examine the functional state of the receptor in the absence of repression (25, 33). As shown in Fig. 5A, a GAL4-RXR LBD fusion is strongly activated by 9-*cis* retinoic acid when expressed in *S. cerevisiae*. As expected, this response to hormone requires the τc domain (compare GAL4RXR225-462 to GAL4RXR225-443). Significant hormone-independent transcription, however, can be detected when a GAL4-RXR LBD fusion is expressed in *S. cerevisiae* (approximately 5% of the hormone-dependent transcription). This hormone-independent transcription, like hormone-dependent transcription, is also eliminated when the τc domain is removed (Fig. 5). Western blotting of cell extracts from *S. cerevisiae* expressing the GAL4 fusions indicates that all fusions are expressed at similar levels (data not shown). The

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ability to detect hormone-independent transcription indicates that in the absence of hormone a small but significant percentage of receptors are in an active conformation. For both hormone-independent and hormone-dependent transcription the τc domain is required for receptors to achieve the active state.

DISCUSSION

In this work TR and RXR have been used to investigate the hormone-mediated transition of nuclear hormone receptors from repressors to activators of transcription. Activation and repression can be mapped to distinct and physically separable domains. Alone, however, neither of the two domains is competent to respond to hormone. Preliminary evidence suggests that the activation and repression domains must be part of the same molecule in order to respond to hormone.

Given that RXR forms heterodimeric complexes with many other nuclear receptors (24, 30), a repression function in RXR could have significant effects on multiple hormonal signaling pathways. Indeed, removal of the τc domain from RXR dramatically reduces hormone-dependent transcription by RXR-RAR, -TR, and -VDR heterodimers. Similar effects of the τc domain deletion have also been observed on RXR-PPAR and RXR-NGFIB heterodimers (data not shown). The negative effect of the RXR τc deletion appears to result from the increased affinity of the heterodimer for members of the recently identified family of TRACs (7, 8, 21, 23) and the failure of hormone to release the corepressor. A similar negative effect on heterodimer activity upon deletion of the RXR τc domain has been observed by Leng et al. (27). In contrast, Valcarcel et al. (42) reported that the RXR τc domain was not necessary for transcription by RXR-RAR heterodimers in vitro. The failure to observe a requirement for the RXR τc domain in vitro may result from the absence of corepressors in the in vitro system or the requirement to preincubate receptors in high concentrations of hormone before initiating the in vitro reaction. These observations suggest that if the affinity of RXR for TRACs can be increased, perhaps in specific cell types or on specific response elements, there may be instances in which heterodimerization with RXR has a negative effect on hormonal signaling. The requirement for the RXR τc domain in transactivation by heterodimers also suggests that binding of hormone by the partner could result in a conformational change in RXR itself.

Interestingly, point mutations in the RXR τc domain have similar although less dramatic effects on transcription and corepressor binding by heterodimers (data not shown). The observation that single point mutations in the RXR τc domain can impact transactivation by heterodimers supports the idea that heterodimers function as integrated units. The data also suggest that it may be possible to allosterically regulate heterodimer activity through ligands that specifically bind to RXR.

The ability to construct receptors with different levels of hormone-independent transcription by combining distinct activation and repression domains suggests a model in which receptors are in an equilibrium between repressing and activating states. The position of the equilibrium is determined by the relative strengths of the two functional domains (Fig. 4). For instance, the combination of a strong repressor with a weak activator (TR) results in a receptor that represses transcription in the absence of hormone. Combining a weak repressor with a strong activator (RXR) results in a receptor that is essentially neutral (Fig. 1). Combining a weak repressor with a weak activator (RXT) or a strong repressor with a strong activator (TRX) results in weak repressors (Fig. 1). Finally, combining a weak repressor with a very strong activator (RXR-VP16) (Fig. 1) (16, 34) results in a constitutively active receptor. Interestingly, Harding and Lazar (18) have shown that the orphan receptor Rev-Erb, which appears to lack a τc domain, is a constitutive repressor. These different states most likely represent different conformations of the LBD. For instance, the repressive state may identify a conformation permissive for repressor binding, while the active state identifies a conformation in which the repressor cannot bind (Fig. 4). For simplicity, the model proposes two states; however, there is no reason to rule out the possibility of multiple states or conformations along a gradient between repression and activation. The requirement for the τc domain in both relief of repression and positive transactivation suggests that this domain is required to initiate and/or maintain the conformational change. Structural analysis supports this hypothesis. In the presence of hormone the τc domains of RAR and TR pack loosely on the surface of the LBD and make contacts with the hormone (35, 43). The possibility that the τc-dependent conformational change uncovers additional positive activation functions also cannot be ruled out.

The equilibrium model described above suggests that the τc domain can be active in the absence of hormone and serves to counterbalance repression. The observation that hormone-independent transactivation requires the τc domain (Fig. 5) supports the hypothesis that hormone receptors can achieve an active state in the absence of hormone. The finding that the τc domain reduces the interaction between SMRT and receptors (Fig. 3) (7) also supports the idea that in the absence of hormone, receptors are not static but exist in an equilibrium shifting between repressive and active states. The model further suggests that the function of hormone is to drive the equilibrium in one direction by destabilizing the repressive state and/or stabilizing the activating state. If the affinities of hormone for receptors in the repressive and active states are identical, then hormone acts by promoting the transition from the repressive state to the active state. The possibility that hormone has a higher affinity for receptors in the active state exists, however. In this case the active state is achieved both by more favorable binding of hormone and by the ability of hormone to promote the transition from the repressive to the active state.

In conclusion, the level of transcription by hormone receptors results from ligand modulating the interplay between distinct repression and activation functions. This finding raises the possibility that other factors also independently regulate the activity of these two domains. Interestingly, several receptors, including RXR, have been shown to be activated by the phosphatase inhibitor okadaic acid (26, 32). The presence or absence of cell-type-specific or developmentally specific *trans*-acting factors or posttranslational modifications adds a level of regulation in addition to hormone that can modulate receptor activity and adds to the complexity of hormonal signaling.

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