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Thyroid Hormone Response Element Organization Dictates the Composition of Active Receptor*

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Thyroid hormone (triiodothyronine, T_3) is known to activate transcription by binding heterodimers of thyroid hormone receptors (TRs) and retinoid X receptors (RXRs). RXR-TRs bind to T_3 response elements (TREs) composed of direct repeats of the sequence AGGTCA spaced by four nucleotides (DR-4). In other TREs, however, the half-sites can be arranged as inverted palindromes and palindromes (Pal). Here we show that TR homodimers and monomers activate transcription from representative TREs with alternate half-site placements. TRβ activates transcription more efficiently than TRα at an inverted palindrome (F2), and this correlates with preferential TRβ homodimer formation at F2 *in vitro*. Furthermore, reconstruction of TR transcription complexes in yeast indicates that TRβ homodimers are active at F2, whereas RXR-TRs are active at DR-4 and Pal. Finally, analysis of TRβ mutations that block homodimer and/or heterodimer formation reveal TRE-selective requirements for these surfaces in mammalian cells, which suggest that TRβ homodimers are active at F2, RXR-TRs at DR-4, and TR monomers at Pal. TRβ requires higher levels of hormone for activation at F2 than other TREs, and this differential effect is abolished by a dimer surface mutation suggesting that it is related to composition of the TR·TRE complex. We propose that interactions of particular TR oligomers with different elements play unappreciated roles in TRE-selective actions of liganded TRs *in vivo*.

Thyroid hormone receptors (TRα[5] and TRβ) modulate gene expression by binding to thyroid hormone response elements (TREs) in target gene promoters (1–5). TR activity is primarily regulated by thyroid hormone (as 3,5,3′-triiodo-L-thyronine (T_3)), which alters the conformation of the receptor C-terminal ligand-binding domain (LBD) to promote dissociation of corepressors and association of coactivators (6). Nevertheless, TR activity is also influenced by the sequence, arrangement, and promoter context of the TRE (2, 3). Liganded TRs activate transcription from some TREs and repress transcription at others (reviewed in Ref. 3 and see Refs. 7 and 8). In addition, TRE sequence and arrangement influence the magnitude of response to unliganded and liganded TRs, T_3 concentration dependence, dominant negative activities of mutants that arise in resistance to thyroid hormone syndrome, and the direction and extent of response to TRβ isoform-selective ligands (9–13). Improved understanding of the molecular basis of these effects could help us to harness these selective activities.

TRs bind to DNA either as heterodimers with the closely related retinoid X receptor (RXR) or as homodimers and monomers, and each species exhibits preferences for different TREs (reviewed in Refs. 1 and 2). TREs are composed of degenerate repeats of the sequence AGGTCA, with the half-sites arrayed as direct repeats spaced by four nucleotides (DR-4), inverted repeats spaced by four to six nucleotides (IPs) or palindromes (Pal). Heterodimers of RXRs and TRs bind each of these elements, with a strong preference for DR-4. In contrast, TRβ homodimers bind strongly to IP elements, weakly to DR-4, and not all to Pal (9, 14–17), and TRα homodimers bind TREs, at best, only weakly (18, 19). Finally, both TRs can bind to DNA elements as monomers. The fact that different oligomeric forms of TR bind to DNA and exhibit clear preferences for different TREs raises the possibility that the nature of the response element could influence TR action and T_3 response by recruitment of distinct TR transcription complexes with unique activities.

It is clear that RXR-TRs are important mediators of T_3 action. TR binding to RXRs *in vivo* has been verified by obser-

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[5] The abbreviations used are: TR, thyroid hormone receptor; T_3, triiodothyronine; RXR, retinoid X receptor; TRE, T_3 response element; IP, inverted palindrome; LBD, ligand-binding domain; DBD, DNA-binding domain; CTE, C-terminal extension; Pal, palindrome; NR, nuclear receptor.

vations that TRs consistently copurify as complexes with RXRs from cell and tissue extracts (1, 3). Moreover, analysis of RXR knock-out mice reveals defective T_3 responses (3, 20), and RXRs and TRs synergize at direct repeats in cotransfection experiments in some cell types (21, 22). Furthermore, native TREs are often comprised of DR-4 elements, which bind preferentially to RXR-TRs (1, 3), and *in vitro* DNA binding properties of RXR-TRs are consistent with roles in T_3 response; RXR-TRs form stable complexes with DNA that persist in the presence of hormone (23–25). Finally, chromatin immunoprecipitation analysis confirms that RXR-TRs occupy DR-4 elements in the *Xenopus laevis* TRβ and thyroid hormone/bZIP promoters at late stages of embryogenesis (26).

Potential roles of other TR oligomers are not as well understood. An RXR gene knock-out fails to affect TR action in ear development, implying that alternate TR species could regulate this process (27). Moreover, the fact that unliganded TRs repress transcription more efficiently at IP elements than DR-4 elements has been attributed to preferential recruitment of homodimers, which bind corepressors more efficiently than RXR-TRs (9, 10, 28–30). Accordingly, it has been shown that TR can bind to an unusual TRE in the human immunodeficiency virus type 1 in the absence of RXR in the frog oocyte chromatin assembly system (31). Roles of alternate forms of TR in T_3 activation are even less clear. Although the fact that TR homodimers dissociate rapidly from cognate TREs and T_3 suppresses homodimer formation on DNA has been taken as an argument against a role for this species in T_3 activation (1), other studies reveal that TR-TR·DNA complexes are stabilized by coactivators (32) and that T_3 response at IP-6 elements is often independent of coexpressed RXR in transfections (9, 33). Most strikingly, a recent study revealed that RXR expression was only required for optimal T_3 response at subsets of positively regulated genes in a mouse preneuronal cell line (34). Thus, there are several indications that alternate TR oligomers contribute to T_3 induction.

Study of the actions of different TR oligomers in mammalian cells is often complicated by endogenous expression of RXR and NR coregulators. To overcome this difficulty, we (35–38) and others (39) have examined TR action in the yeast *Saccharomyces cerevisiae.* This species is devoid of known NRs and NR coregulators and can be used to reconstruct defined TR transcription complexes in a eukaryotic cell background. These studies indicate that TRs indeed activate transcription at selected TREs in the absence of RXR (35, 39) and that the extent of TR homodimer formation to different DR-4 elements *in vitro* correlates with T_3 response (39).

RXR-TR heterodimer and TR homodimer surfaces have also been defined. There are contacts between respective LBDs and DBDs of RXRs and TRs (1), whereas TR homodimer formation at DR-4 and IP-6 relies exclusively on LBD-LBD contacts (14, 40–42). We used x-ray structure-directed mutagenesis to define these surfaces and showed that RXR-TRβ heterodimer and TRβ homodimer formation requires a small hydrophobic patch at the junction of helices (H) 10 and H11 in the LBD (17, 43). This surface (LBD dimer surface) resembles those seen in x-ray structures of other NR LBD heterodimers and homodimers (reviewed in Ref. 2). The TRβ interface (DBD heterodimer surface) that contacts the RXR DBD was detected in an x-ray structure of an RXR·TRβ DBD complex on a DR-4 element (44), and mutation of this surface inhibits RXR-TR heterodimer formation at DR-4 *in vitro* (17). The same structure reveals another difference between TRs and RXRs; the TRβ DBD contains an α-helical C-terminal extension (CTE) that is absent from RXR and mediates additional DNA contacts (44). The requirements for these surfaces in T_3 response *in vivo* are not known.

In this study, we compare actions of wild type and mutant TRs at different TREs in yeast and in mammalian cells. Although the data confirm that RXR-TRs activate transcription at DR-4, they suggest that TRβ homodimers and monomers mediate T_3 response at IP-6 and Pal, respectively. We propose that TRE half-site orientation dictates the composition of the active form of TR *in vivo* and that this effect should be considered in analysis of TR activity and design of selective TR modulators.

MATERIALS AND METHODS

Plasmids—The following plasmids have been described previously: T_3-inducible β-galactosidase reporters for yeast transcription assays, TRE-F2x1, TRE-DR-4x1, and TRE-Palx1 and yeast (YEp56) expression vectors for wild type TRβ, RXR, and GRIP1 (35–38); T_3-inducible reporters containing two copies of each TRE driving luciferase expression and a similar 9-*cis*-retinoic acid-responsive reporter containing two copies of a DR-1 element and mammalian (pCMX) expression vectors for TRβ, TRβL422R, and RXR (17).

New TR mutants reported in these studies (pCMX vectors TRβ4XDBD (D104A, Y117A, R120A, and D177A), TRβ3XLBD (L400R, L422R, and M423R), TRβ4XDBD3XLBD, TRβ T-box deletion (deletion of 6 amino acids from 175 to 180 in the T-box region, -ATDLVL) and the yeast Yep56 vector TRβL422R were created in existing vectors using QuikChange site-directed mutagenesis kits (Stratagene). The presence of the mutation(s) was verified by DNA sequencing using Sequenase kits (Stratagene). FLAG and Myc-tagged TR expression vectors were created by PCR amplification of the TRβ cDNA and insertion into FLAG and Myc vectors (Clontech) at appropriate restriction sites.

Mammalian Cell Culture, Electroporation, and Luciferase Assays—Human promonocyte U937, HEK, HeLa, and U2-OS cells were maintained at 37 °C, 5% CO_2 subcultured in media RPMI 1640 with 10% newborn bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin. Transfections were carried out as described previously (45). After incubation for 24 h at 37 °C with ethanol or T_3, cells were collected by centrifugation, and the pellets were solubilized by addition of 150 μl of 0.25 M Tris-HCl, pH 7.6, containing 0.1% Triton X-100. Luciferase activity was analyzed by standard methods (luciferase assay system, Promega).

Gel Shifts—For Fig. 1, TRs were labeled with [^{35}S]methionine, and complex formation was measured at unlabeled TREs in a 20-μl reaction with 1 μg of poly(dI-dC) (Amersham Biosciences) (17). The binding buffer contained 25 mM HEPES, 50 mM KCl, 1 mM dithiothreitol, 10 μM $ZnSO_4$, 0.1% Nonidet P-40, 5% glycerol. After 30 min at room temperature, the mixture was

loaded onto a 5% nondenaturing polyacrylamide gel that was previously run for 30 min at 200 V. TR and RXR·TR·DNA complexes were visualized as follows: the gel was run at 4 °C for 120–180 min at 200 V in a running buffer containing 45 mM Tris borate, pH 8.0, and 1 mM EDTA, fixed, dried, and exposed to autoradiography. For other figures, binding of TRs and RXR-TRs to DNA was assayed as described previously (43), by mixing 20 fmol of TRs ± RXRs with 300,000 cpm of [γ-^{32}P]ATP-radiolabeled TRE oligonucleotide.

Yeast Strains and Transcriptional Analysis—The *S. cerevisiae* strain YPH499 (*MAT*α, *ura3, lys2, ade2, trp1, his3, leu2*) was used for most transformations. For reporter assays, transformants were grown in minimal medium (0.67% yeast nitrogen base, 2% glucose) supplemented with adenine and lysine (both at 40 mg/liter), as described previously (35–38). The yeast transformants were isolated and grown in the appropriate minimal medium with added supplements as required. Cells were grown overnight with T_3 at a final concentration of 1 μM, harvested, washed, resuspended in Z buffer, and lysed with glass beads (425–600 μm) before centrifugation. The supernatant was collected, and the protein concentration was determined by the Lowry method using bovine serum albumin as a standard. Twenty micrograms of protein were used for the β-galactosidase assay, and transcriptional activities were expressed as Miller units/mg of protein, as described previously (35–38). Data shown were pooled from three independent experiments and calculated as the means ± S.E.

Cell Extracts, Immunoprecipitation, and Western Blotting—HeLa cells were cotransfected with 5 μg of pCMV-Myc-TRβ1 and 5 μg of pCMV-Tag2B-FLAG-TRβ1 by electroporation at 240 V and 950 microfarads. 24 h after transfection, cells were washed with phosphate-buffered saline and harvested. Samples of whole cell extracts were lysed in RIPA buffer (50 mM Tris, pH 8.1, 150 mM NaCl, 0.2% SDS, 1% sodium deoxycholate, 1% Nonidet P-40, 5 mM EDTA) containing protease inhibitor mixture (Calbiochem) and 1 mM phenylmethylsulfonyl fluoride at 4 °C for 1 h, sonicated, and then centrifuged at 20,000 × *g* for 15 min. 100 μl (400 μg of total protein) of supernatant was diluted with 500 μl of phosphate-buffered saline, and 4 μg of rabbit anti-FLAG antibody (Rockland, Inc.) was added. After incubation overnight at 4 °C, 50 μl of protein G-Sepharose beads (Amersham Biosciences) were added to each sample, and the lysates were incubated for 1 h at 4 °C. The beads then were washed six times with TBS-T (Tris-buffered saline; 25 mM Tris, pH 8.3, 192 mM glycine, containing 0.05% Tween 20), suspended in 2× SDS sample buffer containing 5% β-mercaptoethanol, heated for 10 min at 95 °C, and resolved on 8% denaturing polyacrylamide gels. After transferring to Immun-Blot polyvinylidene difluoride membranes (Bio-Rad), the membranes were blocked with 5% nonfat milk in TBS-T and incubated with primary antibody, mouse Myc-TRβ1 antibody (Clontech), at 1:1000 (w/v) dilution in 1% nonfat milk in TBS-T. After washing with TBS-T, immunoblots were incubated with horseradish peroxidase-conjugated goat anti-mouse-IgG (Santa Cruz Biotechnology) in 1% TBS-T, and proteins were visualized using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

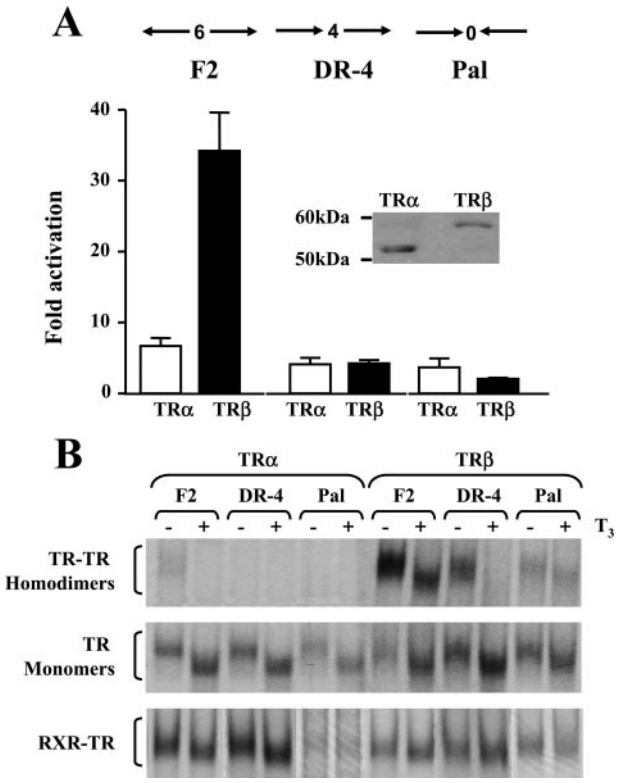

FIGURE 1. **TRβ activates transcription strongly at F2 and binds as a homodimer.** *A*, results of transfection analysis in U2-OS cells, comparing action of both TR isoforms at different TREs. *Inset* shows an image of a nylon membrane blot of an SDS-polyacrylamide gel used to separate transfected cell extracts and probed with an anti-FLAG tag antibody by Western blot. *B*, x-ray autoradiographic image of non-denaturing gels used to separate [^{35}S]methionine-labeled TRs and RXR-TRs in complex with response element oligonucleotides, F2, DR-4, and Pal. Images of TR-TR homodimers and TR monomers are derived from different positions in the same gel. RXR-TR is from a separate gel shift experiment run in parallel. Results are representative of more than five experiments.

RESULTS

TRβ, but Not TRα, Is Superactive at an IP-6 Element (F2)—First, we first compared the actions of two major hormone-binding TR isoforms (TRα1 and TRβ1) at TRE-dependent reporters in cell culture (Fig. 1*A*). TRβ gave significantly larger T_3 responses at an IP-6 element (F2) than TRα, 30–40-fold *versus* 6-fold. By contrast, the two TR isoforms gave comparable T_3 responses at a DR-4 element (4–5-fold), and T_3 responses were weaker with TRβ at Pal (2–3-fold *versus* 4–5-fold for TRα). Western blotting of transfected cell extracts with an antibody against an N-terminal FLAG tag in both TRs revealed similar expression levels (Fig. 1*A, inset*). Moreover, varying the amounts of TR expression vector confirmed that differential effects were not related to differences in amounts of TR required for optimal activation at each element (not shown).

Analysis of DNA binding preferences of TRs and RXR-TRs confirms that TRβ homodimerizes on DNA *in vitro* and that TRα does not (Fig. 1*B*). TRβ homodimers bind strongly to F2 and, to a lesser extent, to DR-4. As expected, T_3 inhibited TRβ homodimer formation at both elements, although significant residual binding of liganded TRβ at F2 was detected in the presence of hormone. In the same gels, TRβ and TRα bound weakly to all three TREs as monomers. Moreover, in parallel, both TRs

formed heterodimers with RXR at all three TREs, with preferential binding to DR-4 (DR-4 > F2 > Pal). Thus, TRβ activates transcription more efficiently at F2 than TRα, and this correlates with enhanced homodimer formation at F2 *in vitro*.

TRβ Homodimers Activate Transcription at F2 in Yeast—To determine the extent to which different TRβ oligomers activate transcription in eukaryotes, we assembled different TR transcription complexes in yeast stably transfected with TRE-dependent reporters and examined the effects of a mutation in the TR LBD dimer surface (TRβL422R) in each context (17).

TRβ homodimers and RXR-TRs were both active in yeast but exhibited distinct TRE preferences (Fig. 2, *A–C*). In accordance with our previous results, TRβ gave weak T_3 response at all three elements, regardless of the presence of coexpressed RXR, and these responses were enhanced by GRIP1 (35). With TRβ and GRIP1, large T_3 responses were obtained at F2, and these were inhibited by the TRβL422R mutation (Fig. 2*A*). By contrast, T_3 responses obtained with TRβ and GRIP1 at DR-4 and Pal were smaller and were enhanced by the same mutation (Fig. 2, *B* and *C*). Thus, TR-TR homodimers are active at F2, whereas homodimer formation places a constraint on T_3 response at other elements. Although RXR did not enhance T_3 response at F2 (Fig. 2*A*), it potentiated T_3 response about 7-fold at DR-4 (Fig. 2*B*) and about 2-fold at Pal (Fig. 2*C*). This RXR-dependent effect required the TRβ LBD dimer surface, as RXR failed to enhance activity of the TRβL422R mutant. Thus, RXR-TRs are active at DR-4, and heterodimer formation provides a moderate advantage for T_3 response at Pal *versus* TR monomers.

It is unlikely that the TRβL422R mutation inhibits T_3 response via indirect effects on hormone binding or cofactor recruitment. TRβL422R does not exhibit reduced affinity for T_3 (17) and bound strongly to GRIP1 and other coactivators *in vitro* (not shown). Thus, our data suggest that TRβ homodimers are active at F2 in yeast and that RXR-TR heterodimers are active at DR-4 and, to some extent, at Pal. In addition, the fact that TRβL422R displays moderate activity at all three TREs in the presence of GRIP1 implies that TR monomers have the potential to activate transcription.

TRE-specific Requirements for TRβ LBD Dimer Surface in Mammalian Cells—Next, we examined the role of the TRβ LBD dimer interface at different TREs in mammalian cells. For these experiments, we compared the effects of transfected TRβ and TRβL422R at different TREs in U937 monocytes, which do not express significant levels of TRs (45). We examined T_3 response with a range of quantities of transfected TRβ and TRβL422R expression vectors to ensure detection of optimal TR activity.

Wild type TRs exhibited a similar activation profile at each TRE (Fig. 3, *A–C*). Peak induction of 30-fold at F2, 15-fold at DR-4, and 7-fold at Pal was obtained with 0.5–1.0 μg of transfected TRs. Moreover, higher levels of TRs led to auto-inhibition (squelching), although the extent of this effect varied. Unliganded TRs do not repress transcription at these minimal promoters to a measurable degree in these conditions (not shown).

In contrast, the effects of the TRβL422R mutation were different at each TRE. TRβL422R exhibited markedly reduced activity relative to wild type TRβ at F2, at all levels of transfected vector (Fig. 3*A*). The mutant also exhibited reduced activity at DR-4, but this defect was overcome with higher amounts of transfected TRβL422R vector (Fig. 3*B*). Finally, TRβL422R activated transcription as efficiently as wild type TR at Pal, and

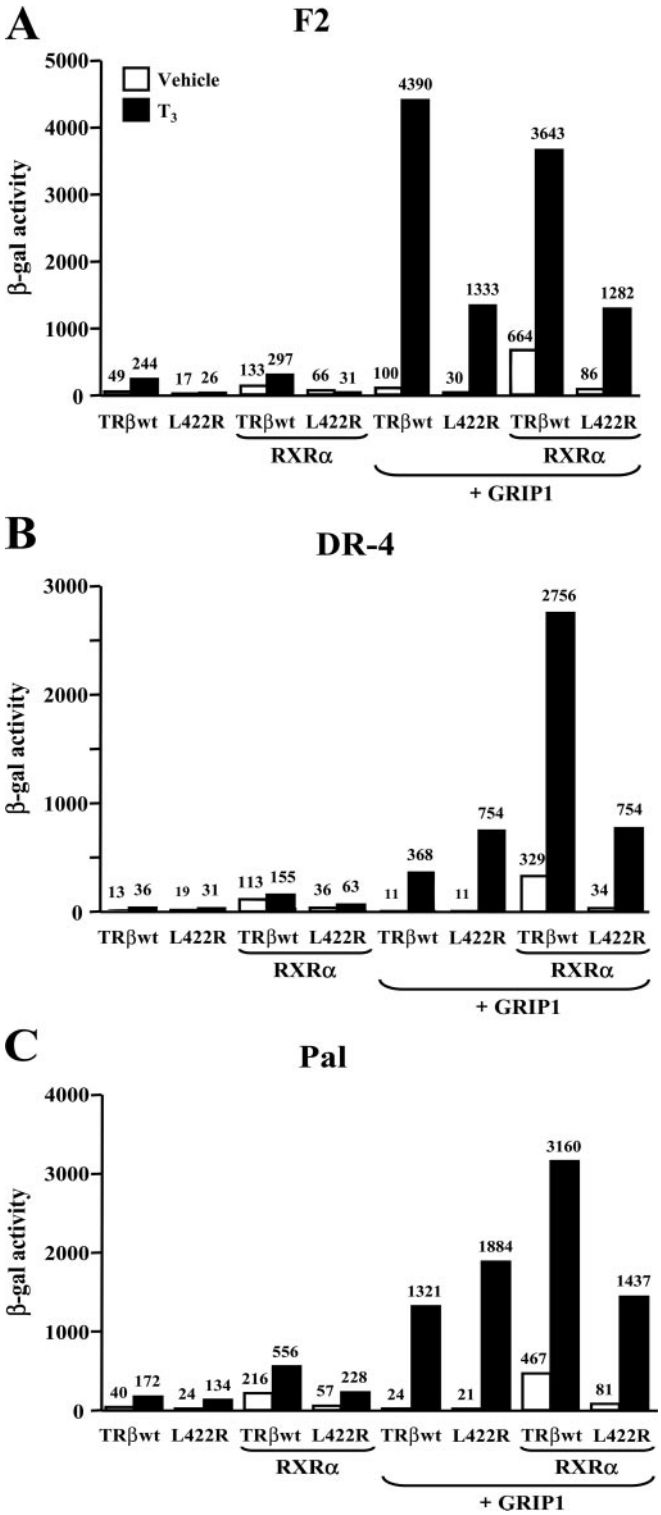

FIGURE 2. **TR homodimers activate transcription at F2 in yeast.** *A* shows β-galactosidase activities detected in extracts of yeast transformants stably transfected with an F2-regulated reporter and, as indicated, TRβ, TRβL422R, RXRα, and GRIP1 and treated with vehicle or 1 mM T_3 (*black bars*). β-Galactosidase activity is expressed as Miller units/mg protein. The data show a single determination that is representative of the average of at least three experiments. *B* and *C*, as in *A*, using a DR-4-regulated reporter (*B*) and Pal-regulated reporter (*C*).

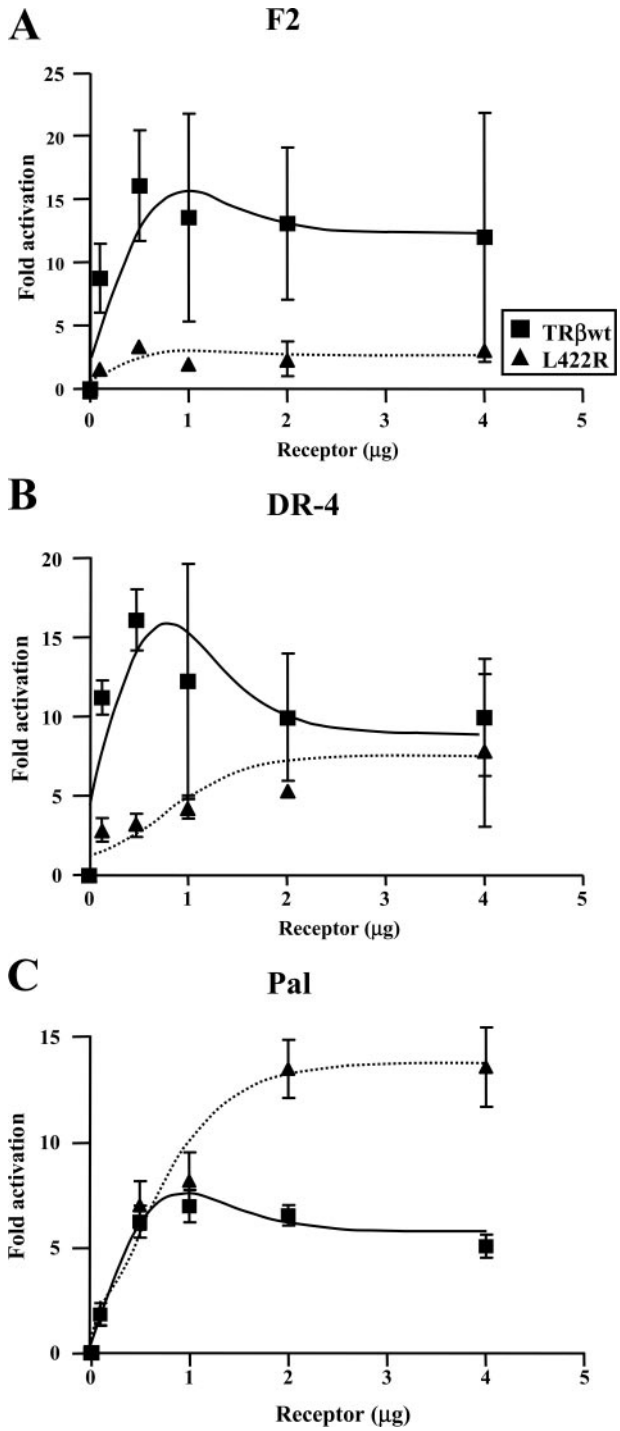

FIGURE 3. **Requirements for the TR LBD dimer surface in mammalian cells.** *A,* F2. U937 cells were cotransfected with 4 μg of F2 luciferase and increasing concentrations (0.1, 0.5, 1.0, 2.0, and 4.0 μg) of TRβ or TRβL422R expression vectors and treated with vehicle or 100 nM T_3. Data represent fold T_3 inductions obtained at each amount of TR expression vector and represent the average of six experiments. *B,* as above, with DR-4-luciferase. Data were from six experiments. *C,* as above, with Pal-luciferase. Data were from four experiments.

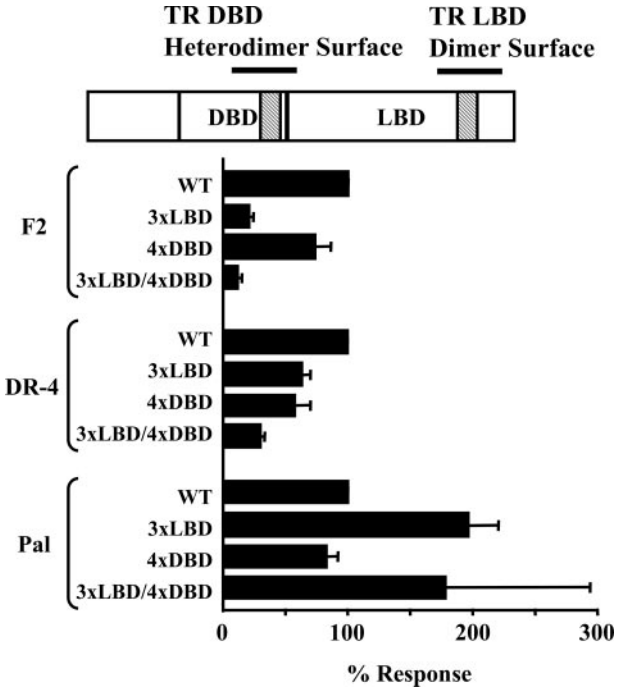

FIGURE 4. **DBD heterodimer surface is required for TR action at DR-4.** Relative positions of TR DBD heterodimer and LBD dimer surfaces are shown in schematic at *top*. *Panels* represent luciferase activities assayed in U937 cells transfected with respective TRE-regulated reporters (4 μg) and TRβ or TRβ mutant expression vectors (2 μg) and treated $\pm 10^{-6}$ M T_3, as in Fig. 4. The data show the average of five experiments. *WT*, wild type.

possibly even more efficiently at higher levels of transfected vector (Fig. 3*C*). Similar results were also obtained with TRβ in several other cell types, including HeLa, HEK, and U2-OS (not shown).

To our knowledge, the data in Fig. 3 represent the first demonstration that the TRβ LBD dimer surface is needed for optimal T_3 response in mammalian cells. Nevertheless, the fact that there are TRE-selective requirements for this surface implies that homodimer or heterodimer formation is required for optimal response at F2 and DR-4 but not at Pal.

TRE-specific Requirements for TRβ DBD Heterodimer Surface—Next, we assessed requirements for the TR DBD surface that contacts the RXR DBD. To do this, we performed transfections, as above, with vectors for TRs bearing mutations at key residues in the DBD heterodimer surface (4XDBD) (44), alone or in combination with a triple Arg substitution affecting hydrophobic residues in the LBD dimer surface (3XLBD) (17). We used relatively high levels of transfected TRs (2 μg) to highlight different requirements for the LBD dimer surface at different TREs; in these conditions, TRβL422R exhibits reduced activity at F2, significant residual activity at DR-4, and elevated activity at Pal (see Fig. 3).

Contributions of the DBD heterodimer surface and LBD dimer surface vary at each TRE. TR action at F2 was absolutely dependent upon the LBD dimer surface (TRβ 3XLBD), and mutation of the DBD heterodimer interface had little effect (Fig. 4*A*). By contrast, optimal TR action at DR-4 requires both interfaces (Fig. 4*B*), mutation of the DBD heterodimer surface (TRβ 4XDBD) or LBD dimer surface (TRβ 3XLBD) inhibited TR activity by 35%, and mutation of both interfaces (TRβ 4XDBD and 3XLBD) inhibited TR activity by 70%. Finally, neither interface was required for T_3 response at Pal (Fig. 4*C*).

Several conclusions can be derived from these studies. First, the TR LBD dimer surface is required for T_3 response at F2, but the DBD heterodimer surface is not. Thus, TR homodimers are active or RXR-TRs activate transcription in a configuration that

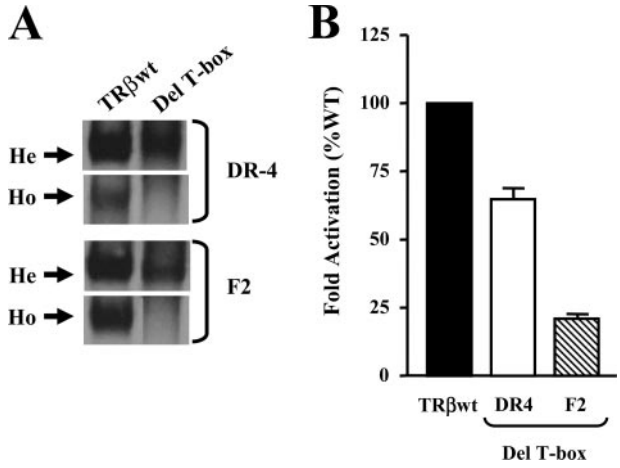

FIGURE 5. **Deletion of the TRβ T-box blocks homodimer formation on DNA and inhibits TR action at F2.** *A* shows a gel shift assay comparing binding of TRβ and TRβ T-box deletion mutant to DR-4 and F2, as homodimers (*Ho*) and heterodimers (*He*) with RXR. *B,* results of transfection analysis comparing TRβ and TRβ T-box deletion mutant activity at DR-4 and F2 elements in U937 monocytes, average of six experiments.

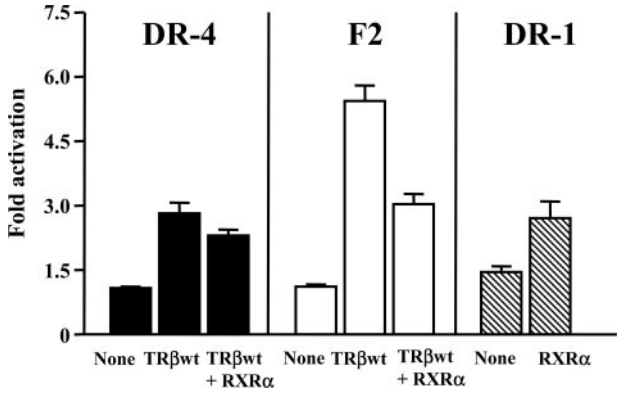

FIGURE 6. **RXR inhibits T_3 response at F2.** Fold T_3 activation obtained at different TREs in the presence of transfected TRβ ± RXR in U937 monocytes. In parallel, RXR action at a cognate DR-1-driven reporter is shown.

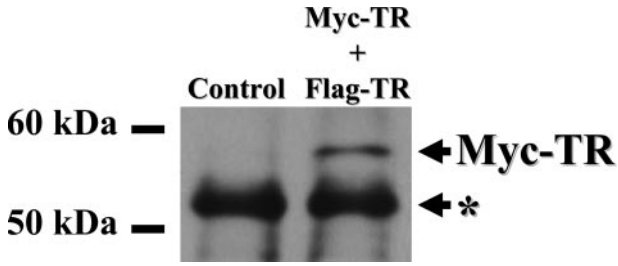

FIGURE 7. **TR self-associates in transfected cells.** Blot of an SDS-polyacrylamide gel used to separate extracts of cells transfected with FLAG ± Myc-tagged TRs and probed with an anti-Myc antibody after immunoprecipitation with anti-FLAG antibody is shown. The 52-kDa species marked with an *asterisk* is present in nontransfected cell extracts and does not correspond to TR.

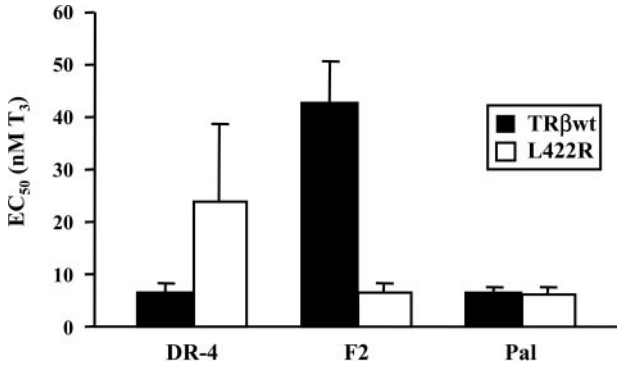

FIGURE 8. **Differential T_3 concentration sensitivity at different reporters is reversed by a dimer surface mutation.** Average dose of T_3 that is required for half-maximal activation (EC_{50}) at DR-4, F2, and Pal in U937 cell transfections performed with 2 μg of TRβ or TRβL422R expression vector.

does not rely upon DBD-DBD contacts. Second, TR action at DR-4 requires both surfaces, consistent with the notion that RXR-TRs are active, with their respective DBDs engaged in interactions similar to those in the RXR-TR DBD x-ray structure (44). Finally, the fact that T_3 response at Pal is independent of both interfaces suggests that homo- and heterodimer formation is not needed for T_3 response at this element and that monomers are active.

Evidence for TRβ Homodimer Activity and Formation in Mammalian Cells—The TRβ DBD CTE, which makes auxiliary contacts with DNA (44), was required for homodimer formation and T_3 response at F2. Deletion of part of the TRβ DBD CTE (the T-box) abolished TR homodimer formation at F2 and DR-4, but only weakly inhibited RXR-TR heterodimer formation (Fig. 5*A*). In transfections, the T-box deletion inhibited T_3 response at F2, where homodimer formation is preferred, but exhibited a lesser effect at DR-4, where RXR-TR formation is preferred (Fig. 5*B*).

Transfected RXR was not compatible with T_3 response at F2 (Fig. 6). RXR did not affect T_3 response at DR-4, but inhibited T_3 response at F2 by about 50%. Similar results were also obtained in other cell types (not shown). RXR did enhance the activity of a reporter that contained an RXR response element (DR-1) in the presence of RXR ligand (9-*cis*-retinoic acid), confirming that it is expressed in functional form. The fact that RXR overexpression reduces T_3 response at F2 represents further suggestive evidence that RXR-TRs are not active at this element and that TR-TR homodimers are active.

We also confirmed that TR can self-associate in transfected cells. We introduced expression vectors for TRs with in-frame N-terminal FLAG and Myc peptide tags into U2-OS cells. Fig. 7 shows that the TRβ Myc epitope is detectable after precipitation of transfected cell extracts with an anti-FLAG antibody. This finding indicates that TR-TR interactions can occur in living cells.

Mutation of the LBD Dimer Surface Affects T_3 Sensitivity—Finally, we examined the relationships between requirements for the LBD dimerization surface at different TREs and response element-selective effects on T_3 dose response. Analysis of the amounts of T_3 required for the half-maximal response at each TRE confirms that TRβ action at F2 exhibits a higher EC_{50} than at DR-4 (9) and extends these studies to show that similar amounts of T_3 are required for half-maximal response at DR-4 and Pal (Fig. 8). The same data show that mutation of the TRβ LBD dimer surface enhanced T_3 sensitivity at F2 and reduced T_3 sensitivity at DR-4. Thus, interactions at the LBD dimer surface influence T_3 dose response even though this mutation does not affect the affinity of TR for T_3 (17). Because RXR-TR heterodimers are active at DR-4, TR monomers at Pal, and TRβ homodimers at F2, we propose that different TR oligomers exhibit differential sensitivity to T_3 in mammalian cells, with RXR-TRs and TR monomers > TR homodimers.

DISCUSSION

The findings reported in this study indicate that RXR-TRs are not the only species of TR that is capable of transducing T_3 signals in eukaryotic cells; homodimers and monomers also play a role. We stress that our data support the notion that RXR-TRs are important players in T_3 response (1–4, 25, 46–53). RXR-TRs mediate T_3 activation at DR-4 in yeast, and T_3 activation at DR-4 in mammalian cells is uniquely dependent on the LBD dimer surface and the TR DBD heterodimer surface, consistent with the notion that heterodimers are active (Figs. 3 and 4). This does not seem to be the case at other TREs. TRβ activates transcription strongly at F2 in mammalian cells, but TRα does not, correlating with the extent of homodimer formation *in vitro*. Moreover, TRβ homodimers are active at F2 in yeast; TRβ activates transcription from this element in the presence of GRIP1 and the absence of RXR, and these effects require the LBD dimer surface. Finally, in mammalian cells: 1) TRs exhibit a strong requirement for the LBD dimer surface at F2, excluding the possibility that monomers are involved; 2) a TRβ T-box deletion that strongly inhibits homodimer formation but not heterodimer formation on DNA selectively inhibits TR action at F2; and 3) RXR inhibits T_3 response at F2, suggesting that heterodimer formation inhibits T_3 response at this element. Together, these findings suggest that TR-TR homodimers are active at F2, and accordingly, TR-TR homodimer formation can be detected in coimmunoprecipitation experiments with tagged TRs from extracts of living cells.

There may also be roles for monomers. A mutant version of TR that only forms monomers, TRβL422R, activates transcription with moderate efficiency at all three TREs in the presence of GRIP1 in yeast, and almost as efficiently RXR-TRs at Pal in this cell type. Furthermore, T_3 response at Pal in mammalian cells is completely independent of the LBD dimer and DBD heterodimer surfaces.

Our results complement recent findings of another group (34). Here, knockdown of endogenous RXR expression with specific short interfering RNA only impaired transcription of a subset of T_3-regulated genes in a murine preneuronal cell line, suggesting that RXR requirements of endogenous genes are quite variable and that other TR species must be active.

Although the data reveal significant correlation between DNA binding preferences of different TR oligomers and their respective roles in transcriptional activation from different response elements, our data also suggest that unknown influences must favor TRβ homodimer and monomer interactions with their preferred TREs in mammalian cells. The fact that TRβ homodimers and RXR-TRs activate transcription from F2 and DR-4 in yeast and mammalian cells and the ability of TR monomers to activate transcription at Pal in mammalian cells correlates well with observed DNA binding preferences of each species *in vitro*. Nevertheless, RXR-TRs predominate in mammalian cell extracts, bind a variety of TREs *in vitro*, and even activate transcription at Pal in yeast, so it is somewhat puzzling that RXR-TRs only appear able to activate from the DR-4 element in mammalian cell transfections. One obvious explanation is that TR transfection alters the TR/RXR ratio to highlight effects of homodimers and monomers. We find, however, that the TRβ CTE mutant that is defective in homodimer formation exhibits impaired T_3 response at low levels of transfected receptor (Fig. 5) (data not shown) and that even low amounts of TRs function as monomers at Pal (Fig. 3). Thus, actions of TRβ homodimers and monomers seem to be favored at particular TREs over the predominant RXR-TR species by unspecified mammalian cell factors. Perhaps stabilizing effects of coregulators on TR DNA binding activity contribute to this effect (32).

What is the physiological importance of our findings? Although we believe that it is likely that RXR-TRs mediate many or even most T_3 responses in living cells, our data raise the interesting possibility that alternate TR species play special roles at subsets of TREs and that these influences could expand the range and complexity of physiological responses to T_3. For example, the fact that TR-TR homodimers are active at F2, coupled with results described herein and in previous publications (9), suggests that this species must be both resistant to hormone activation and highly active in the presence of saturating levels of T_3. Thus, TRβ homodimers may confer a large dynamic range of responses to high levels of T_3 on particular subsets of target genes with F2-like elements. Our data have not yet revealed similar selective actions for TR monomers; T_3 responses at Pal are qualitatively similar to those at DR-4, but it remains possible that TRE-selective actions of monomers will also be observed at some Pal elements or at variant TREs with single AGGTCA half-sites (54).

We recognize that our findings only indicate that contributions of different oligomeric forms of TR are possible and that direct proof that RXR-TRs, TR-TR homodimers, and TR monomers are active at particular genes in particular contexts awaits further analysis. It will be particularly instructive to bring chromatin immunoprecipitation to bear on this question. It is already established that RXR-TRs occupy promoters of T_3-activated genes with DR-4 elements in the frog but that TRs can occupy an unusual human immunodeficiency virus type 1 in the absence of RXRs in a frog oocyte chromatin assembly assay (26, 31). It nevertheless remains important to examine this question in the context of mammalian genes. It may be possible to determine whether variable RXR dependence of endogenous T_3-responsive genes in mouse preneuronal cells correlates with the lack of RXR recruitment to target promoters or particular TRE sequence organization (34). More generally, it will be interesting to determine the degree of overlap between chromosomal TR- and RXR-binding sites using a chromatin immunoprecipitation approach combined with nested oligonucleotides that span the genome, as demonstrated with estrogen receptors (55).

Finally, we stress that our studies do not exclude the possibility that the TRE sequence could influence TR activity in other ways. Half-site placement could influence TR activity via effects on TR conformation; it is known that TR-TR homodimers bind quite strongly to subsets of DR-4 elements (39), and examination of likely orientations of the TR DBDs at IP-6 and DR-4 elements indicates that one DBD must swivel to contact DNA at DR-4 (1). These conformational adaptations

could exert profound influences on TR activity. Variations in half-site spacing could also play a role (22, 56). Altered half-site spacing can reverse the direction of retinoid response from activation to repression by altering the polarity of RXR-retinoic acid receptor pairs (57). Although analogous effects have not been seen for TRs, it is known that differences in half-site spacing of inverted palindromic TREs do affect ligand response. The SERCa2 promoter contains a DR-4 element that is important for T_3 activation (TRE1) and two auxiliary inverted palindromic elements (TRE2, IP-4 and IP-6, TRE3) (12). When TR activity is assayed in isolation at the SERCa2 IPs, T_3 activates transcription from TRE3 but not TRE2, and the TRβ isoform-selective modulator GC-1 exhibits strong aberrant antagonist activity at TRE3 and weak antagonist activity at TRE2. Finally, DNA contacts are known to exert allosteric influences on nuclear receptor DNA binding domains that can then be propagated to neighboring transactivation functions (58). Thus, degenerate TRE sequences could influence receptor conformation and activity, even within the context of a standard TRE organization.

In summary, however, our findings confirm that the effects of TRE half-site placement on TR oligomer recruitment is at least one important factor that should be considered in the regulation of T_3 responsiveness *in vivo*. It is noteworthy that unusual actions of TRβ isoform-selective modulators have been detected at IP elements (11, 12); GC-1 represses transcription from IP elements in the SERCa2 promoter, and GC-24 is specifically superactive at F2. It will be interesting to examine the possibility that these effects are related to preferential homodimer recruitment, and whether it will be possible to develop TRE-selective modulators by targeting drugs toward particular TR species.

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