Thyroid hormone (TH) actions are mediated by nuclear receptors (TRs α and β) that bind triiodothyronine (T3, 3,5′,3′-triiodo-L-thyronine) with high affinity, and its precursor thyroxine (T4, 3,5,3′,5′-tetraiodo-L-thyronine) with lower affinity. T2 contains a bulky 5′ iodine group absent from T3. Based on the x-ray crystal structures of the TR ligand binding domain (LBD), we have predicted that TH analogues with 5′ substituents should fit poorly into the ligand binding pocket and perhaps behave as antagonists. We therefore examined how T2 affects TR activity and conformation. We obtained several lines of evidence (ligand dissociation kinetics, migration on hydrophobic interaction columns, and non-denaturing gels) that TR-T2 complexes adopt a conformation that differs from TR-T3 complexes in solution. Nonetheless, T2 behaves as an agonist in vitro (in effects on coregulator and DNA binding) and in cells, when conversion to T3 does not contribute to agonist activity. We determined x-ray crystal structures of the TRβ LBD in complex with T3 and T4 at 2.5-Å and 3.1-Å resolution. Comparison of the structures reveals that TRβ accommodates T2 through subtle alterations in the loop connecting helices 11 and 12 and amino acid side chains in the pocket, which, together, enlarge a niche that permits helix 12 to pack over the 5′ iodine and complete the coactivator binding surface. While T2 is the major active TH, our results suggest that T2 could activate nuclear TRs at appropriate concentrations. The ability of TR to adapt to the 5′ extension should be considered in TR ligand design.

Thyroid hormone (TH) plays important regulatory roles in metabolism, homeostasis, and development by binding and altering the transcriptional regulatory properties of two related nuclear receptors (NRs), the thyroid hormone receptors (TRs) α and β (1, 2). Most TH produced in the thyroid gland is secreted in the form of thyroxine (T4; 3,5,3′,5′-tetraiodo-L-thyronine) (2, 3). The thyroid gland also produces smaller amounts of triiodothyronine (T3; 3,5,3′-triiodo-L-thyronine) and reverse T3 (rT3; 3′,3′,5′-triiodo-L-thyronine), and 80% of T4 is converted to T3 and rT3 in peripheral tissues by two selenium deiodinases, which are tissue-specific (4). Current beliefs are that T3 is the dominant active form of TH; T3 binds the TRs with an affinity about 20–30 times higher than that of T4 (5–9), and some studies suggest that T3 is present at higher concentrations in the nucleus than T4 (10, 11). Nonetheless, the question of whether T3 is simply a prohormone or an active TH species is not completely resolved. T4 exerts rapid nongenomic effects at several loci distinct from TRs (12). Moreover, saturating levels of T4 activate transcription of TH-responsive genes in cell culture (see for example Ref. 5). Whereas it is possible that at least some of this activity is due to T3 generated from T4 in the cell, these results suggest that T3 may act as a TR agonist. Normal concentrations of plasma-free T3 are about 4–6-fold higher than those of T3 (19 pmol/liter of T3 versus 3.3 pmol/liter T3) and intracellular T4 and T3 levels can differ because of variations in uptake and T2 to T3 conversion (3); thus, it is conceivable that intracellular T3 in some context could occupy a significant fraction of nuclear TRs.

If T2 does behave as an agonist, then it should bind to TR in a similar way to T3 and induce conformational changes in the TR similar to those induced by T3 (13, 14). T3 interacts with the TR ligand binding domain (LBD), located in the receptor C terminus. The x-ray crystal structure of TRα or TRβ complexed with T3 reveals that hormone is completely enclosed in a ligand binding pocket within the core of the LBD. It is thought that the enclosure is due to ligand-induced packing of the LBD C-terminal helix 12 (H12) against the LBD; a rearrangement that also disrupts the corepressor binding surface and completes the coactivator binding surface, leading to exchange of coregulators and influence on gene expression in vivo (15).

Unlike T3, T4 possesses a bulky iodine substituent at the 5′-position of the first thyronine ring. X-ray crystal structures have been determined for TR-LBDs complexed with several different high affinity agonists, including T3a, Dimit (3, 5-dimethyl-3′-isopropyl-L-thyronine), and the TRβ-specific ligands GC-1 (3,5 dimethyl-4′(4-hydroxy-3′-isopropylbenzyl)-phenoxy acetic acid), and KB141 (3,5-dichloro-4′(4′-hydroxy-3′-isopropylphenoxy)phenyl] acetic acid) (16–18). In each of these cases,
the agonist contains a 5’ hydrogen group that lies close to the inner surface of H12. We therefore predicted that compounds with bulky side groups would perturb the folding of H12 against the body of the LBD and exploit this feature to create TR antagonists based on the notion that 5’ extensions would preclude appropriate H12 packing and coactivator binding (19–24). For example, addition of a 5’ isopropyl group, similar in size to an iodine group, to the agonist MIBRT (3,5-dibromo-4(3’isopropyl-4’-hydroxyphenoxy)benzoic acid) creates the TR antagonist DIBRT (3,5-dibromo-4(3’,5’-diisopropyl-4’-hydroxyphenoxy)benzoic acid) (22). Thus, it is conceivable that T4, with a 5’ iodine extension, could even behave as an antagonist in some settings. Improved understanding of the way that the TRs adapt to the 5’ iodine group will be therefore important for understanding T4 action and key principles of NR antagonist design.

In this study, we examine T4 interactions with TR, the way that T4 influences TR activity in vitro and in cells in culture and determined the x-ray crystal structure of TR in complex with T4. We find that the TR-T4 complex is less stable than the TR-T3 complex, and that T4-ligated TRs exhibit properties that are similar to unliganded TRs in solution. Nonetheless, T4 behaves as an agonist in cell-free assays and transfected cells. The x-ray structure of the TR LBD-T4 complex reveals that a previously undetected niche in the ligand binding pocket widens, relative to the size of the pocket observed in the TR-LBD-T3 complex, to accommodate the 5’ iodine, permitting H12 to pack against the LBD surface in the presence of the larger ligand. Thus, the enclosed TR hormone binding pocket accommodates T4 without complete disruption of overall TR-LBD structure. These results suggest that T4 will act largely as a TR agonist if present at high enough concentrations in the nucleus.

MATERIALS AND METHODS

Thyroid Hormone Binding Assay—Thyroid hormone binding and analog competition assays were performed as previously described (9). Kd values were calculated by fitting saturation curves and competition data to the equations of Swillens (25) using the GraphPad Prism program (GraphPad Software, San Diego, CA).

Hydropathic Interaction Chromatography of TR—TR-LBDs were expressed in E. coli and partially purified on phenyl-TOyopearl, TSK-DEAE, TSK-heparin, and TSK-phenyl columns without TH as described previously (9). For each analog tested, TR was incubated for 1 h with a 5-fold molar excess of the analog relative to the final TR concentration and, where appropriate, radiolabeled T4 in complex with the TR was present at high enough concentrations in the nucleus.

GST Pull-down Assay—Labeled TRs were expressed using a TNT-coupled transcription translation kit. GRIP1 (amino acids 563–1121) (26), TRAP220(622–701) (27), and N-CoR(1944–2453) (28) were prepared in Escherichia coli BL21 as a fusion protein with glutathione S-transferase as per the manufacturer’s protocol (Amersham Biosciences). Binding experiments were performed by mixing glutathione-linked and radiolabeled TRs containing 4 μg of GST fusion proteins (Coomassie Plus protein assay reagent, Pierce) with 1–2 μl of 7S-labeled TR in 150 μl of binding buffer (20 mM HEPES, 150 mM KCl, 25 mM MgCl2, 10% glycerol, 1 mM diethiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, protease inhibitors, and 20 μg/ml bovine serum albumin) for 1.5 h. Beads were washed three times with 200 μl of binding buffer, and bound proteins were separated using 10% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography.

Electrophoretic Mobility Shift Assays—In vitro translated TR was produced in reticulocyte lysates, TNT T7 Quick (Promega), and 20 floods of translated receptor were incubated with 300,000 cpm of [γ-32P]ATP-radiolabeled P2 oligonucleotides and 1 μg of poly(dI-dC) (Amersham Biosciences) in a 20-μl volume (29–31). The binding buffer contained 25 mM HEPES, 50 mM KCl, 1 mM dithiothreitol, 10 μM ZnSO4, 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. To separate TR-DNA complexes, the gel was run at 4 °C for 120–180 min at 200 V, using a running buffer containing 45 mM Tris borate (pH 8.0), and 1 mM EDTA.

Reporter Cells—The assay procedure, described previously (22), utilized Chinese hamster ovary cells stably expressing TRa1 and TRβ1 containing a stably integrated reporter gene with a single TRE (DR-4) cloned into the position of the mouse mammary tumor virus promoter hormone response element, driving expression of alkaline phosphatase coding sequences.

Crystallographic and Data Collection—The TR8 LBD was purified for crystallization trials using cobalt affinity and hydrophobic interaction chromatography first without and then with hormone as previously described (18). Crystals of the TR complex were obtained by the hanging drop method, with a 10.5 mg/ml protein stock solution and mother liquor consisting of 100 mM sodium cacodylate and 900 mM sodium acetate, pH 7.2. Crystals were cryoprotected by immersion in sequential baths of 100 mM sodium cacodylate and 1.1 mM sodium acetate, pH 7.2, with 3, 8, and 15% glycerol. Crystals were subjected to a final swipe through a bath with 25% glycerol before flash-freezing in liquid nitrogen.

Crystals of the TR complex were obtained similarly, with a crystallization mother liquor of 100 mM sodium cacodylate and 700 mM sodium acetate, pH 7.4. Use of extremely fresh protein and microseeding with T3-crTR-LBD crystals (> 0.1% of the final crystal) were found to be essential to obtaining diffraction quality crystals. Crystals were then cryoprotected using sequential glycerol baths as for the T3 complex, but with a mother liquor of 100 mM sodium cacodylate and 900 mM sodium acetate, pH 7.4. Crystals were analyzed at the Advanced Light Source synchrotron facility, beamline 5.02. For the T3 complex, 15° of data were collected with 1.5° oscillations; for the T4 complex, 75° of data were collected with 0.5° oscillations. Reflections were indexed in DENZO and scaled in SCALEPACK.

Structural Refinement of the T4 Complex—A molecular replacement solution was found using EPMR, employing the wild-type TR8/TRIAC structure with hormone omitted as a probe. The structure was then subjected to multiple rounds of simulated annealing, followed by positional and B-factor refinement in CNS. Occupancies were refined in CNS for atoms constituting 95% of the model. Refinement was then continued using REFMAC of the CCP4 suite. In the final stages of refinement, water molecules were added to the structure both manually and in ARP/WARP. Refinement steps alternated with manual rebuilding steps in Quanta98 and O, guided by F–F and 2F–F maps calculated using FFT of the CCP4 suite, and a simulated annealing omit map calculated using CNS.

Structural Refinement of the T4 Complex—The T4 data set was subjected to molecular replacement in EPMR and simulated annealing and positional, B-factor, and occupancy refinement in CNS as for the T3 complex. In light of the markedly higher resolution of the data for the T4 complex, the refined T4 structure was then least squares fitted in O to the model of the T3 complex. A composite model consisting of protein from the fitted T3 complex and ligand from the T4 complex was created and used for subsequent refinement against the T4 complex data.

To guard against model bias, this composite model was subjected to simulated annealing and positional refinement in CNS. However, this treatment raised Rp, markedly, as compared with Rp of the composite after positional refinement. This indicates that the true structure of the T3 complex is close to the T4 complex model used to create the composite. Subsequent refinement and model rebuilding were carried out as described above for the T4 complex.

RESULTS

The TR-T4 Complex Is Less Stable Than the TR-T3 Complex and Adopts a Different Conformation in Solution—We first investigated interactions of TRs with T3 and T4 (Fig. 1A). We previously determined that T3 has an affinity for TRs (Kd = 0.06 nM) about 30-fold higher than T4 (Kd = 2 nM) (6). T4 also dissociates from TRs more rapidly than T3 (Fig. 1, B and C). Whereas it took 8.4 and 6.2 h for half the T3 to dissociate from in vitro translated preparations of TRa and TRβ at 4 °C, it only took about 0.15 h and 0.06 h for half of the T4 to dissociate from TRa and TRβ, respectively. Thus, TRs form a complex with T4 that is significantly less stable than the TR-T3 complex.

We next examined elution of T4-ligated TRs from TSK-
phenyl hydrophobic interaction columns (HIC), an assay that detects ligand-dependent conformational alterations in TR-LBDs and provides a crude index of hydrophobicity (9). In accordance with previous results (9), liganded TR\textsubscript{α}/H9251-LBDs eluted ahead of unliganded TRs (Fig. 2A). Moreover, TR\textsubscript{α} preparations in complex with several agonists T\textsubscript{3}, Triac (3,3',5'-triiodothyroacetic acid), 3'-IPT\textsubscript{2} (3'-isopropyl-3,5-diiodo-L-thyronine), and Dimit eluted nearly together, with elution order paralleling relative affinities of ligand for TR (T\textsubscript{3} > Triac > IPT\textsubscript{2} > Dimit). By contrast, the TR\textsubscript{α}-T\textsubscript{4} complex eluted closer to unliganded TR. Similar results were also obtained with the TR\textsubscript{β}/H9252-LBD; the TR\textsubscript{β}-T\textsubscript{4} complex eluted from the column between the TR\textsubscript{β}-T\textsubscript{3} complex and unliganded TR\textsubscript{β}/H9252 (Fig. 2B).

It is unlikely that the unusual elution profile of the TR-T\textsubscript{4} complex is related to the low affinity of T\textsubscript{4} for TRs; the TR\textsubscript{α}-Dimit complex eluted at a similar position to other TR agonist complexes even though Dimit binds TR\textsubscript{α} with an affinity five times lower than T\textsubscript{3} ($K_D = 9$ nM for Dimit versus $2$ nM for T\textsubscript{3}). It is also unlikely that the unusual elution profile is related to rapid T\textsubscript{4} release during passage over HIC. Continuous dissociation of T\textsubscript{4} would lead to a broad and not a discrete symmetric peak as observed here, although the “shoulder” observed with the TR\textsubscript{β}-T\textsubscript{4} complex may reflect T\textsubscript{4} dissociation (Fig. 2B). Nonetheless, we directly examined migration of [\textsuperscript{125}I]T\textsubscript{4} prebound to TRs on HIC (Fig. 2C). Radiolabeled T\textsubscript{4} migrated at the same position as the TR\textsubscript{α}-T\textsubscript{4} complex, whereas free T\textsubscript{4} did not elute from the column in these timescales (not shown), confirming that TR remains bound to T\textsubscript{4} as it passes over HIC. Thus, the unique elution profile of the TR-T\textsubscript{4} complex reflects an unusual conformation that exposes more hydrophobic surface than TRs in complex with T\textsubscript{3} or other analogues.

\section*{Modulator Binding Properties of TR-T\textsubscript{4} Complexes Resemble Those of TR-T\textsubscript{3} Complexes}

We next determined whether T\textsubscript{4} behaved as an agonist under cell-free conditions. Fig. 3A shows that T\textsubscript{4} and T\textsubscript{3} promoted equivalent levels of TR binding to bacterially expressed nuclear receptor interacting regions of the coactivators GRIP1 and TRAP220. T\textsubscript{4} and T\textsubscript{3} also showed comparable activity in promoting binding of radiolabeled full-length GRIP1 and TRAP220 to bacterially expressed TR\textsubscript{β}-LBD (not shown). Finally, both ligands promoted TR release from bacterially expressed preparations of the receptor-interacting region (C terminus) of the corepressor, N-CoR (Fig. 3B). Thus, T\textsubscript{4} and T\textsubscript{3} behave as agonists in cell-free conditions.

TR agonists promote near complete dissociation of TR dimers, but not RXR-TR heterodimers, from DNA response
elements (TREs) containing half-sites aligned as inverted palindrome (F2/IP-6) or direct repeats (DR-4) (29, 30). T4 and T3 both promoted TRα homodimer release from an F2/IP-6 element and enhanced interactions of TR monomers with the same element (Fig. 4). Both forms of TH only modestly reduced RXR-TRα heterodimer binding in the same conditions. Similar results were also obtained using DR-4, and with TRβ and both TREs (not shown). Nonetheless, TR migration was slower in the presence of T4 than T3 (this was most evident for the monomer). Thus, T4 resembles T3 in terms of regulation of DNA binding activity, but TR-T4 and TR-T3 complexes exhibit different mobilities, underscoring the idea that TR-T4 complexes adopt a different structure from TR-T3 complexes in solution (see Fig. 2).

**T4 Behaves as a TR Agonist in Cell Culture**—We next examined the behavior of T4 in cell culture. T4 elicited a similar maximal response to T3 in cultured Chinese hamster ovary cells that were stably transfected with a TH-regulated reporter gene and a vector that expresses either TRα or TRβ (TRANβ cells, Fig. 5A) or TRβ (TRANβ cells, Fig. 5B) (22, 32). In both cases T4 exhibited a potency that is about 10% that of T3. It is unlikely that T4 to T3 conversion accounts for the activity of administered T4 in these conditions for several reasons. Treatment of TRα and both agonist forms of TH (Fig. 6). The other regions include: N-terminal residues 199–212 (part of the DNA binding domain C-terminal helix (H0), which is included in this structure); H2 residues 234–243, portions of the underlying β-sheet (residues 318–321 and 325–339) and the loop between H2 and H3; and the N terminus of H3 (residues 248–267). Each of these regions of TR usually exhibits poor electron density in crystals, suggesting that they correspond to mobile regions of the protein (16–18). Thus, alterations in these regions are less likely to be significant for understanding ligand discrimination than those of H11-H12 region.

The H11-H12 loop (residues 446–453) is shifted by about 1 Å in the TR-T4 structure relative to the TR-T3 structure (Fig. 7), the C terminus of H11 (residues 437–444) is pulled inward toward the pocket, accentuating a kink also present in the T3 structure and other agonist-bound TR LBD structures, and the C-terminal end of H12 (residue 460) is pushed outwards in the presence of T4. Despite these alterations, residues that comprise the coactivator binding surface (on H12 and the upper part of H3 and H5) adopt a structure with backbone positions identical to those seen in the TR-T3 structure, and side chain positions nearly identical. This is consistent with the finding that T4 promotes coactivator binding in vitro, and displays agonist activity in vivo. Nonetheless, direct comparisons in Rasmd indicate that H12 (residues 452–460) has closer contacts with the main body of the LBD in the TR-T3 complex than in the TR-T4 complex. This suggests that H12 packs less tightly against the LBD. Moreover, the average B-factor for protein atoms was higher for TR-T4 (54.05) than for TR-T3 (49.61), and the TR-T4 structure had lower resolution (3.1 Å) than the TR-T3 structure (2.5 Å). Thus, the TRβ-T4 complex exhibits a greater degree of disorder than the TR-T3 structure.

The conformational alterations that occur within the hormone binding pocket near the T4 5′ idoine group are shown in detail in Figs. 8 and 9. Strikingly, the 5′ idoine fits neatly into a small “niche” in the wall of the pocket (Fig. 8A). This feature is analogous to similar niches that accommodate the other idoine groups in the T4 and T3 structures and is comprised of two distinct parts: an upper region that consists of residues
from several static helices that line the pocket of the LBD (Ile276 on H3, and Met310, Met313 on H6), and a lower region comprised of His435 on H11, and Phe455 and Phe459 on H12. This precisely positioned niche permits TR to accommodate T₄ completely within the enclosed pocket despite the presence of the 5’ iodine group. The niche is also present within the TRβ-T₃ structure, but it is smaller (Fig. 8B). Superimposition of the TR-T₄ and TR-T₃ complexes reveals this region of the pocket expands slightly in the presence of T₄ (Fig. 9, compare mesh surface, T₄ with solid surface, T₃). This expansion is a result of a number of amino acid side chain shifts. The largest involves Met310 (on H6), which lies above the 5’ iodine in the TR-T₄ complex. If one considers the receptor in the orientation seen in Fig. 9, a steric clash between Met310 and the 5’ iodine shifts the entire ligand toward the “left” of the receptor relative to the position of ligand in the TRβ-T₃ complex (detailed in Table II). This repositioning accentuates further steric clashes between the 5’ iodine group and side chains of two residues (Phe455, Phe459) on H12 itself. In addition, the kink in H11 probably results from a steric clash between the 5’ iodine and His435 (see Table II for distances). These alterations enlarge the niche that accommodates the 5’ iodine substituent and permit H12 to pack against the LBD and complete the coactivator binding surface.
probably related to the bulky 5'-iodine moiety that, based on our previous structures of TR-LBDs in complex with T3 and related agonists, should not fit readily into the hormone binding pocket (14, 16–18). Indeed, placement of some bulky 5' extensions on high affinity TR agonists can even create antagonists (21). Thus, we asked how T4 interacts with TR, whether it behaves as an agonist or antagonist, and how it can fit into the TR ligand binding pocket.

We initially examined properties of TR-T4 complexes. We confirmed that T4 bound to TR more weakly than T3, and further demonstrated that T4 dissociates from TRs faster than T3, (Fig. 1). Moreover, the TR complex with T4 is less compact than that with T3, as suggested by migration of TR-T4 complexes closer to unliganded TRs than to TR-agonist complexes on HIC (Fig. 2, A and B) and in gel shift assays with DNA (Fig. 4). The unusual HIC elution profile is not a reflection of lower affinity of TR for T4, because TR complexes with Dmit (which lacks a 5' substituent yet only exhibits 20% of the affinity of T4 for TR) elute at a similar position to TR-T3 complexes (Fig. 2). It is also unlikely to reflect rapid dissociation of T4 while on the column, because TRs in complex with radiolabeled T4 also elute at a similar position to TRs in complex with unlabeled T4 (Fig. 2C). Despite the less compact nature of the TR-T4 complex, maximally effective doses of T4 were as effective as those of T3 in stimulating association of coactivators (GRIP1 and TRAP220; Fig. 3), release of corepressors (N-CoR and SMRT; Fig. 3) and dissociation of TR homodimers from DNA (Fig. 4). Moreover, maximally effective doses of T4 were as effective as those of T3 in stimulating activity of a TRE-regulated reporter (Fig. 3) and dissociation of TR homodimers from DNA (Fig. 4). Thus, it is likely that T4 and T3 promote similar overall conformational rearrangements within the TR-LBD in these conditions and that H12 must fold into the active conformation in the presence of T4.

The crystal structure of the TRβ-T4 complex supports the notion that T4 induces a TR conformation similar to that observed with higher affinity agonists (Fig. 6). TR adopts this fold because, overall, T4 fits tightly into the ligand binding pocket despite the presence of the 5’-iodine group. The pocket accommodates the bulky 5’-iodine via shifts in the position of several amino acid side chains in the pocket relative to their positions in the TR-T3 complex. These changes enlarge a niche that lies close to the 5’-position of the first thyronine ring and closely matches the size and shape of the 5’-iodine (Figs. 8 and 9). The pocket accommodates the bulky 5’-iodine via shifts in the position of several amino acid side chains in the pocket relative to their positions in the TR-T3 complex. These changes enlarge a niche that lies close to the 5’-position of the first thyronine ring and closely matches the size and shape of the 5’-iodine (Figs. 8 and 9). The requirements for these structural alterations for fitting of T4 relative to T3 likely explain the reduced affinity of the TRs for T4 relative to T3. However, the niche permits H12 to fold over the bulky iodine group and complete the coactivator binding surface. Thus, the presence of an adaptable niche in the TR ligand binding pocket allows T4 to behave as agonist, despite the 5’-extension.
Although H12 adopts the typical active conformation in the presence of T₄, our crystal structures indicate that the H11-H12 loop is more mobile and more loosely packed against the LBD in the presence of T₄ than in the presence of T₃ (Figs. 6 and 7). These features suggest explanations for the observed differences between the behavior of TR-T₄ and TR-T₃ complexes. A tendency of H12 to oscillate between conformations would reduce the efficiency of the pocket and allow T₄ to dissociate more readily. Loose packing of H12 would also expose more of the hydrophobic interior of the protein, explaining unusual mobilities of the TR-T₄ complex in HIC and gel shifts. While T₄ consistently behaves as a full agonist in our hands, it is conceivable that the loose packing of H12 induced by T₄ versus T₃ could allow the TR to open to external influences that alter the response to the ligand. For example, in cells with high corepressor and/or low coactivator levels, H12 might be forced into the unliganded conformation and T₄ could display partial agonist, or even antagonist, activity. This issue will require further investigation.

We do not yet have a similar structure of TRs in complex with T₄, but there are great overall similarities between the TR isoforms in terms of overall LBD fold (18), sequence and, as we have learned that many other TR ligands with extensions (22–24), interfering with H12 packing or occupying the pocket without “extensions.”

We previously proposed that TR ligands with bulky 5’ side chains should perturb H12 and act as antagonists (reviewed in Ref. 21). This idea, the extension hypothesis (19, 20), has been partially validated by our synthesis of novel TR antagonists based on these principles (22–24), and structures of other NRs in complex with antagonists (such as selective estrogen receptor modulators) (34, 35). Nonetheless, T₄ acts as an agonist, just as we have learned that many other TR ligands with extensions that are even bulkier than the T₄ 5’ iodine group can behave as agonists (23, 24). Thus, the nature of the extension and its relationship to the rest of the ligand is important for overall agonist/antagonist activity, and TR must accommodate larger ligands in ways that cannot be easily predicted from structures of TR ligands without “extensions.”

NR antagonists perturb H12 position in two ways, by directly interfering with H12 packing or occupying the pocket without inducing the structural changes required for the agonist configuration (reviewed in Ref. 21 and references therein). Our studies add to an emerging pattern, which suggests that NRs alter their conformations in a variety of ways to accommodate hormone analogs and allow them to act as agonists. The TR-T₄ crystal structure reported here reveals that the pocket can reorganize to accommodate the 5’ iodine group, but with a resulting strain of the overall structure relative to T₃. An extreme case of accommodation is for PXR, where the pocket expands to accommodate larger ligands and collapses to accommodate smaller ligands (36–38). In this case, packing does not appear to result in the stability differences we have detected between T₄ versus T₃-ligated TRs. Finally, we recently showed that TR accommodates a ligand (GC-24) that binds TRβ with about 40-fold the affinity of TRα, and has a 3’ phenyl extension and a 5’ hydrogen (39), by opening up a hydrophobic patch on the inner surfaces of H3 and H11 that is not normally part of the pocket. It will be interesting to determine how TR accommodates ligands with even bulkier 5’ extensions (22–24), or why ligands with particular 5’ extensions, such as the DI-BRT isopropyl group and the NH₃ phenyl group, act as antagonists.

Our studies do not address the question of whether T₄ is a relevant species of TH in physiological settings. As indicated in the Introduction, many factors regulate relative T₃/T₄ concentrations in the nucleus, making it difficult to gauge the extent to which intracellular T₄ participates in TR binding. Nonetheless, the observation that TR can reorganize to create a niche that precisely accommodates the T₄ 5’ iodine, coupled with the fact that the potency of T₄ is about 10% that of T₃ in cell culture and that free circulating T₄ concentrations are 4–6-fold those of T₃, raises the distinct possibility that T₄ could exhibit significant agonist activity in humans.
34. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**, 927–937
35. Brzozowski, A. M., Pike, A. C., Dauter, Z., Hubbard, R. E., Bonn, T., Engstrom, O., Ohman, L., Greene, G. L., Gustafsson, J. A., and Carlquist, M. (1997) *Nature* **389**, 753–758
36. Watkins, R. E., Davis-Searles, P. R., Lambert, M. H., and Redinbo, M. R. (2003) *J. Mol. Biol.* **331**, 815–828
37. Watkins, R. E., Maglich, J. M., Moore, L. B., Wisely, G. B., Noble, S. M., Davis-Searles, P. R., Lambert, M. H., Kliewer, S. A., and Redinbo, M. R. (2003) *Biochemistry* **42**, 1430–1438
38. Watkins, R. E., Wisely, G. B., Moore, L. B., Collins, J. L., Lambert, M. H., Williams, S. P., Willson, T. M., Kliewer, S. A., and Redinbo, M. R. (2001) *Science* **292**, 2329–2333
39. Borngraeber, S., Budny, M. J., Chiellini, G., Cunha-Lima, S. T., Togashi, M., Webb, P., Baxter, J. D., Scanlan, T. S., and Fletterick, R. J. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 15358–15363