THYROID HORMONE RESPONSE ELEMENT ORGANIZATION DICTATES THE COMPOSITION OF ACTIVE RECEPTOR

Lara Franciele Ribeiro Velasco1*, Marie Togashi1,2*, Paul G. Walfish3, Rutinéia de Paula Pessanha1, Fanny Nascimento Moura1, Gustavo Barcelos Barra1, Phuong Nguyen2, Rachelle Rebong2, Chaoshen Yuan, Luiz Alberto Simeoni1, Ralf Carvalho Justiniano Ribeiro18, John D. Baxter2, Paul Webb2^, Francisco de Assis Rocha Neves1^

From the 1Molecular Pharmacology Laboratory, Department of Pharmaceutical Sciences, School of Health Sciences, University of Brasilia, Brasília, DF, Brazil. 2Diabetes Center and Department of Medicine, University of California, San Francisco, CA 94143, USA and 3Department of Medicine, Endocrine Division, Mount Sinai Hospital, University of Toronto Medical School, Toronto, ON, Canada, M5G 1X5.

Running Title: Thyroid Hormone Receptor Dimerization

*These authors contributed equally to the work. ^RCJR - Deceased

^Correspondence to: F. A. R. Neves, M.D., Ph.D., Laboratorio de Farmacologia Molecular, Departamento de Ciências Farmacêuticas, Universidade de Brasília, Caixa Postal – 4473, 70919-970 -Brasília -DF Brazil, tel. +55 61 3072098, fax +55 61 3474622, e-mail: chico@unb.br and to P. Webb Ph.D., Diabetes Center & Department of Medicine, University of California, San Francisco CA94122. USA tel 1-415-476-6789, fax 1-415-564-5813, e-mail pwebb@diabetes.ucsf.edu.

Thyroid hormone (triiodothyronine, T₃) is known to activate transcription by binding to heterodimers of thyroid hormone receptors (TRs) and retinoid X receptors (RXRs). RXR-TRs bind preferentially to T3 response elements (TREs) comprised of direct repeats of the sequence AGGTCA spaced by four nucleotides (DR-4). In other TREs, however, the AGGTCA half-sites can be arranged as inverted palindromes (IP) and palindromes (pal). Here, we present evidence that TR homodimers and monomers activate transcription from representative TREs with alternate half-site placements. We find that TRβ activates transcription more efficiently than TRα at an IP element (F2) and that this correlates with preferential TRβ homodimer formation at F2 in vitro. Further, 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 effects of TRβ mutations that block homodimer and/or heterodimer formation reveals 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 an LBD dimer surface mutation suggesting that it is related to the composition of the TR/TRE complex. We propose that interactions of particular TR oligomers with different elements play an unappreciated role in TRE-selective actions of liganded TRs in vivo. Thyroid hormone (TH) receptors (TRα 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 TH (as 3,5,3'-triiodo-L-thyronine, T₃), which alters the conformation of the receptor C-terminal ligand-binding domain (LBD) to promote dissociation of co-repressors 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 (3) and see (7,8)). In addition, TRE sequence and arrangement influences the magnitude of response to unliganded and liganded TRs, T₃ concentration-dependence, dominant negative activities of mutants that arise in resistance to thyroid hormone syndrome (RTH) and the direction and extent of response to TRβ isofrom 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 (1,2)). TREs are comprised 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 T3 response by recruitment of distinct TR transcription complexes with unique activities.

It is clear that RXR-TRs are important mediators of T3 action. TR binding to RXRs in vivo has been verified by observations that TR variants consistently copurify as complexes with RXRs from cell and tissue extracts (1,3). Moreover, analysis of RXR knockout mice reveals defective T3 responses (3,20) and RXRs and TRs synergize at direct repeats in cotransfection experiments in some cell types (21,22). Further, 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 T3 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 TH/bZIP promoters at late stages of embryogenesis (26).

Potential roles of other TR oligomers are not as well understood. An RXR gene knockout 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 1 (HIV1) in the absence of RXR in frog oocyte chromatin assembly system (31). Roles of alternate forms of TR in T3 activation are even less clear. While the fact that TR homodimers dissociate rapidly from cognate TREs and T3 suppresses homodimer formation on DNA has been taken as an argument against a role for this species in T3 activation (1), other studies reveal that TR-TR/DNA complexes are stabilized by coactivators (32), and that T3 response at IP-6 elements is often independent of co-expressed RXR in transfections (9,33). Most strikingly, a recent study revealed that RXR expression was only required for optimal T3 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 T3 induction.

Study of 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 yeast, S. Cerevisiae. This species is devoid of known NRs and NR coregulators and can be used to reconstruct defined TR transcription complexes in an 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 T3 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 (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.
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 T3 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. While the data confirm that RXR-TRs activate transcription at DR-4, they suggest that TRβ homodimers and monomers mediate T3 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. T3 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). T3 inducible reporters containing two copies of each TRE driving luciferase expression and a similar 9-cis RA responsive reporter acid 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, D177A), TRβ3XLBD (L400R, L422R, 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 Quick Change 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 cells, HEK, HeLa and U2-OS cells were maintained on 37°C, 5% CO2 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 previously described (45). After incubation for 24h at 37°C with ethanol, or T3, cells were collected by centrifugation and the pellets were solubilized by addition of 150 µl 0.25 M Tris-HCl, pH 7.6 containing 0.1% Triton X-100. LUC activity was analyzed by standard methods (Luciferase Assay System, Promega).

Gel Shifts - For Fig. 1, TRs were labeled with 35S-methionine and complex formation was measured at unlabeled TREs in a 20µl reaction with 1µg of poly(dI-dC) (Amersham Pharmacia Biotech) (17). The binding buffer contained 25mM HEPES, 50mM KCl, 1mM DTT, 10µM ZnSO4, 0.1% NP-40, 5% glycerol. After 30 min at room temperature, the mixture was loaded onto a 5% non-denaturing 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 for autoradiography. For other Figures, binding of TRs and RXR-TRs to DNA were assayed as previously described (43), by mixing 20 fmols of TRs +/-RXRs with 300,000 CPM of [32P]-γATP radiolabeled TRE oligonucleotide.

Yeast Strains and Transcriptional Analysis - The S. Cerevisiae strain YPH499 (MATα, 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 previously described (35-38). Data shown were pooled from
three independent experiments and calculated as a mean ± SE.

**Cell Extracts, Immunoprecipitation, and Western blotting** - HeLa cells were cotransfected with 5µg of pCMV-Myc-TRβ1 and 5µg pCMV-Tag2B-Flag-TRβ1 by electroporation at 240V and 950µA. 24 h after transfection, cells were washed with PBS 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 cocktail (Calbiochem) and 1 mM phenylmethylsulfonyl fluoride at 4°C for 1h, then sonicated and centrifuged at 20,000g for 15 min. 100µL (400µg total protein) of supernatant was diluted with 500µL of PBS and 4µg of rabbit anti-flag antibody (Rockland, Inc.) were added. After incubation overnight at 4°C, 50µL of protein G sepharose beads (Amersham) were added to each sample, and the lysates were incubated for 1h at 4°C. The beads then were washed six times with TBS-T (Tris Buffered Saline; 25mM Tris pH8.3, 192mM glycine, containing 0.05% Tween 20), suspended in 2xSDS sample buffer containing 5% b-mercaptoethanol, heated for 10 min at 95°C, and resolved on 8% denaturing polyacrylamide gels. After transferring to Immun-Blot PVDF 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% non-fat milk in TBS-T. After washing with TBS-T, immunoblots were incubated with horseradish-peroxidase conjugated goat-anti-mouse-IgG (Santa Cruz, Inc.) in 1% TBS-T, and proteins were visualized using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham).

**RESULTS**

**TRβ, but not TRα, is Superactive at an IP-6 element (F2)**

First, we first compared actions of two major hormone binding TR isoforms (TRα1 and TRβ1) at TRE-dependent reporters in cell culture (Fig. 1A). TRβ gave significantly larger T₃ responses at an IP-6 element (F2) than TRα; 30-40 fold vs. 6-fold. By contrast, the two TR isoforms gave comparable T₃ responses at a DR-4 element (4-5 fold) and T₃ responses were weaker with TRβ at Pal (2-3 fold vs. 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. 1A inset and not shown). 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. 1B). TRβ homodimers bind strongly to F2 and, to a lesser extent, to DR-4. As expected, T₃ inhibited TRβ homodimer formation at both elements; though 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 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. 2A-C). In accordance with our previous results, TRβ gave weak T₃ response at all three elements, regardless of the presence of co-expressed RXR, and these responses were enhanced by GRIP1 (35). With TRβ and GRIP1, large T₃ responses were obtained at F2 and these were inhibited by the TRβL422R mutation (Fig. 2A). By contrast, T₃ responses obtained with TRβ and GRIP1 at DR-4 and Pal were smaller and were enhanced by the same mutation (Fig. 2B, C). Thus, TR-TR homodimers are active at F2, whereas homodimer formation places a constraint on T₃ response at other elements. While RXR did not
enhance T3 response at F2 (Fig. 2A), it potentiated T3 response about seven fold at DR-4 (Fig. 2B) and about two fold at Pal (Fig. 2C). 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 T3 response at Pal vs. TR monomers.

It is unlikely that the TRβL422R mutation inhibits T3 response via indirect effects on hormone binding or cofactor recruitment. TRβL422R does not exhibit reduced affinity for T3 (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 effects of transfected TRβ and TRβL422R at different TREs in U937 monocytes, which do not express significant levels of TRs (45). We examined T3 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. 3A-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 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, 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. 3A). The mutant also exhibited reduced activity at DR-4, but this defect was overcome with higher amounts of transfected TRβL422R vector (Fig. 3B). Finally, TRβL422R activated transcription as efficiently as wild type TR at Pal, and possibly even more efficiently at higher levels of transfected vector (Fig. 3C). 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 T3 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. 4A). By contrast, optimal TR action at DR-4 requires both interfaces (Fig. 4B): 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, 3XLBD) inhibited TR activity by 70%. Finally, neither interface was required for T3 response at Pal (Fig. 4C).

Several conclusions can be derived from these studies. First, the TR LBD dimer surface is required for T3 response at F2, but the DBD heterodimer surface is not. Thus, TR homodimers are active or RXR-TRs activate transcription in a configuration that 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 T₃ response at Pal is independent of both interfaces suggests that homo- and heterodimer formation is not needed for T₃ 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 T₃ 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. 5A). In transfections, the T-box deletion inhibited T₃ response at F2 where homodimer formation is preferred, but exhibited a lesser effect at DR-4, where RXR-TR formation is preferred (Fig. 5B).

Transfected RXR was not compatible with T₃ response at F2 (Fig. 6). RXR did not affect T₃ response at DR-4, but inhibited T₃ response at F2 by about 50%. Similar results were also obtained in other cell types (not shown). RXR did enhance activity of a reporter that contained an RXR response element (DR-1) in the presence of RXR ligand (9-cis RA), confirming that it is expressed in functional form. The fact that RXR overexpression reduces T₃ 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 T₃ Sensitivity

Finally, we examined relationships between requirements for the LBD dimerization surface at different TREs and response element selective effects on T₃ dose response. Analysis of amounts of T₃ required for half-maximal response at each TRE confirms that TRβ action at F2 exhibits a higher EC₅₀ than at DR-4 (9) and extends these studies to show that similar amounts of T₃ 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 T₃ sensitivity at F2 and reduced T₃ sensitivity at DR-4. Thus, interactions at the LBD dimer surface influence T₃ dose response even though this mutation does not affect the affinity of TR for T₃ (17). Since 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 T₃ in mammalian cells, with RXR-TRs and TR monomers>TR homodimers.

DISCUSSION

The findings reported in this paper indicate that RXR-TRs are not the only species of TR that is capable of transducing T₃ 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 T₃ response (1-4,25,46-53). RXR-TRs mediate T₃ activation at DR-4 in yeast and that T₃ 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 T₃ response at F2, suggesting that heterodimer formation inhibits T₃ 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 co-immunoprecipitation 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. Further, T3 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, knock down of endogenous RXR expression with specific siRNA only impaired transcription of a subset of T3 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.

While 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 \textit{in vitro}. Nevertheless, RXR-TRs predominate in mammalian cell extracts, bind a variety of TREs \textit{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 T3 response at low levels of transfected receptor (Fig. 5 and 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 physiological importance of our findings? While we believe that it is likely that RXR-TRs mediate many or even most T3 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 T3. 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 T3. Thus, TRβ homodimers may confer a large dynamic range of response to high levels of T3 on particular subsets of target genes with F2-like elements. Our data have not yet revealed similar selective actions for TR monomers, T3 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 (ChIP) to bear on this question. It is already established that RXR-TRs occupy promoters of T3 activated genes with DR-4 elements in the frog but that TRs can occupy an unusual HIV-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 T3 responsive genes in mouse preneuronal cells correlates with 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 ChIP 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 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). While 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 T3 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; T3 activates transcription from TRE3 but not TRE2 and the TRβ isofom 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 which can then 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 effects of TRE half site placement on TR oligomer recruitment is, at least, one important factor that should be considered in regulation of T3 responsiveness in vivo. It is noteworthy that unusual actions of TRβ isofom 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 towards particular TR species.
REFERENCES

ACKNOWLEDGEMENTS

We thank Dr. F. Schaufele for critical reading of the manuscript and Y-F Yang and R.Y. Wang for yeast assay technical assistance. This work was supported by Brazilian Research Council (CNPq/PADCT SBIO 620003/02-2 and CNPq 40.00.43/02-5); NIH Grants to JDB (DK41482; DK51281, DK61468); Canadian Institutes of Health Operational Grant MOP-49448 and the Mount Sinai Hospital Foundation of Toronto and Department of Medicine Research Funds, the Julius Kuhl and Temmy Latner/Dynacare Family Foundations (to P.G.W.). JDB is consultant to Karo Bio AB, a Biotechnology company with commercial interests in nuclear receptors.
FIGURE LEGENDS

**Fig. 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-PAGE gel used to separate transfected cell extracts and probed with an anti-Flag tag antibody by western blot.

**Fig. 2** TR homodimers activate transcription at F2 in yeast. **A.** Panel shows β-gal activities detected in extracts of yeast transformants stably transfected with a F2 regulated reporter and, as indicated, TRβ, TRβL422R, RXRα and GRIP1 and treated with vehicle or 1 mM T3 (black bars). β-gal activity is expressed as Miller units/mg of protein. The data show a single determination that is representative of the average of at least 3 experiments. **B, C.** As in Fig. 2A, using a DR-4 regulated reporter (B) and Pal regulated reporter (C).

**Fig. 3** Requirements for the TR LBD Dimer Surface in Mammalian Cells. **A.** F2. U937 cells were co-transfected with 4 g of F2 LUC and increasing concentrations (0.1, 0.5, 1.0, 2.0, 4.0 and 8.0 µg) of TRβ or TRβL422R expression vectors and treated with vehicle or 100nM T3. Data represent fold T3 inductions obtained at each amount of TR expression vector and represent the average of 6 experiments. **B.** As above, with DR-4-LUC. Data from 6 experiments. **C.** As above, with Pal-LUC. Data from 4 experiments.

**Fig. 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 LUC activities assayed in U937 cells transfected with respective TRE-regulated reporters (4 µg) and TRβ or TRβ mutant expression vectors (2 µg) and treated +/- 10^{-6}M T3, as in Fig. 4. The data show the average of 5 experiments.

**Fig. 5.** Deletion of the TRβ T-box blocks homodimer formation on DNA and inhibits TR action at F2. **A.** Panel shows a gel shift assay comparing binding of TRβ and TRβ T-box deletion mutant to DR-4 and F2, as homodimers and heterodimers 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.

**Fig. 6.** RXR inhibits T3 response at F2. Fold T3 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.

**Fig. 7.** TR self associates in transfected cells. Panel is an image of a blot of an SDS-PAGE 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. The 52Kd species marked* is present in non-transfected cell extracts and does not correspond to TR.

**Fig. 8.** Differential T3 concentration sensitivity at different reporters is reversed by a dimer surface mutation. Average dose of T3 required for half-maximal activation (EC_{50}) at DR-4, F2 and Pal in U937 cell transfections performed with 2µg TRβ or TRβL422R expression vector.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Thyroid hormone response element organization dictates the composition of active receptor

J. Biol. Chem. published online February 20, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M610700200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2007/02/20/jbc.M610700200.citation.full.html#ref-list-1