Thyroid hormone receptors (TRs) are transcription factors that bind to thyroid hormone response elements (TREs) in the regulatory regions of target genes. TRs are thought to activate transcription primarily as heterodimers with retinoid X receptors (RXRs), with RXR binding upstream to the two directly repeated half-sites in a typical TRE. However, given that TRs and RXRs prefer to bind to different DNA sequences (T/A/G)AGGTCA and GGGGTCA, we postulate that only certain TREs require RXR-TR heterodimerization, depending on the TRE sequence. We have tested this hypothesis by comparing in Saccharomyces cerevisiae the functional activity of TR ± RXR on 10 naturally occurring mammalian TREs. S. cerevisiae was used as a model system because yeast lack endogenous nuclear receptors and thus can be manipulated to express TRs and/or RXRs. We first studied ligand-independent reporter gene activation, which reflects the activity of the activator function 1 (AF-1) domain. The 10 TREs formed a continuous spectrum from being fully dependent on RXR for TF A1 activity to being essentially independent of RXR. Relative independence of RXR generally was seen when the TRE upstream half-site has a TA or TG 5 to the core hexamer. Gel mobility shift assays revealed that functional independence of RXR correlates with the strong binding of TR alone, whereas more RXR dependence correlates with higher binding of RXR-TR heterodimers. Restoration of ligand-dependent (AF-2 domain) reporter gene activation was achieved by expression of the coactivator TIF2. This ligand-induced stimulation was stronger in the presence of TR alone than with RXR plus TR, suggesting a preference for TIF2 activation of TR homodimers. Overall, the data support the notion that the TRE sequence plays an important role in determining the nuclear hormone receptor and coactivator requirements for TR action.

Thyroid hormone receptors (TRs) are ligand-activated tran-

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‡ The abbreviations used are: TR, thyroid hormone receptor; T₃, 3,5,3′-triiodo-L-thyronine; AF-1, activation function 1; LBD, ligand binding domain; AF-2, activation function 2; TRE, thyroid hormone response element; RXR, retinoid X receptor; triac, 3,5,3′-triiodothyroacetic acid; GST, glutathione S-transferase; TIF2, transcriptional intermediary factor-2; EMSA, electrophoretic mobility shift assay; ruMHC, rat α-myosin heavy chain; rUCP, rat uncoupling protein 1; transcription factors that mediate the biological effects of thyroid hormone (T₃). TRs, along with the receptors for steroid hormones, retinoids, and vitamin D, belong to the nuclear hormone receptor superfamily (1). Like other nuclear receptors, TRs exhibit a modular structure with separable functional domains. A ligand-independent transcription activation function (AF-1) is located in the highly variable N-terminal A/B domain (2). The highly conserved DNA binding domain containing two zinc finger motifs is centrally located and characterizes the members of the superfamily (1). The moderately conserved C-terminal ligand binding domain (LBD) possesses a strong receptor dimerization interface and a ligand-dependent activation function, AF-2 (3–5).

TRs exert their effects on gene expression through direct interaction with specific DNA sequences known as thyroid hormone response elements (TREs), usually found in the 5′-flanking regions of T₃-responsive genes. Unlike steroid hormone receptors, TRs interact with TREs even in the absence of ligand. TREs usually are composed of two or more receptor-binding hexameric half-sites related to the sequence AGGTCA arranged as direct repeats (6) or, less commonly, everted repeats (7).

Although TRs are capable of binding to TREs without auxiliary factors, they preferentially bind to many TREs as heterodimers with retinoid X receptors (RXRs) (8, 9). Based on this observation, it is thought that TRs activate gene expression primarily as heterodimers with RXR (reviewed in Refs. 1, 10, and 11). However, the physiological role of RXR in TR action remains to be elucidated, largely because RXR-null mammalian cells do not exist. It has been shown that TRs and RXRs prefer to bind to different DNA sequences T(A/G)AGGTCA and GGGGTCA (12, 13), respectively. Therefore, we postulate that certain TREs are primarily responsive to TR, whereas others require RXR-TR heterodimers for gene activation. To test this hypothesis, Saccharomyces cerevisiae was used as a model system. S. cerevisiae is devoid of endogenous nuclear receptors and thus can be manipulated to express TRs and/or RXRs, which allows for the analysis of receptor requirements dictated by the TRE for TR-mediated gene expression.

Transcriptional regulation by TRs or other nuclear receptors is accomplished by the concerted action of an array of coregulatory proteins including coactivators and corepressors (14). In mammalian cells, the binding of unliganded TRs to “positive” TREs results in repression of transcription, which is mediated by interaction of the TR with a corepressor complex (15, 16). The binding of ligand triggers conformational changes in the TR LBD that result in release of the corepressor complex and recruitment of coactivators (17, 18), thereby leading to gene activation. Little is known about the potential influence of TRE

rGH, rat growth hormone; hPL, human placental lactogen; rME, rat melic enzyme; rP450, rat NADPH:cytochrome P450 oxidoreductase.
sequence on coactivator function. Yeast lack endogenous proteins homologous to mammalian corepressors and coactivators and thus provide a null background that should be advantageous for gaining insight into this issue.

In the present report, 10 naturally occurring mammalian TREs were studied in a yeast model system to test the importance of RXR for the transcriptional activity of TR. The data demonstrate that thyroid hormone response element sequence plays an important role in determining the nuclear hormone receptor and coactivator requirements for TR action.

**EXPERIMENTAL PROCEDURES**

**Yeast Reporter Strains, Expression Plasmids, and Site-directed Mutagenesis**—Yeast strains bearing chromosomally integrated reporter genes were constructed as described previously (19). In brief, double-stranded oligonucleotide response elements were inserted into the EagI site upstream of a basal cytomegalovirus promoter (cyc1) linked to a β-galactosidase reporter gene in the yeast integrating plasmid pS3305. The constructs were confirmed by sequencing and were integrated into the chromosome of S. cerevisiae. The TREs were studied in a yeast model system to test the importance of RXR for the transcriptional activity of TR. The data demonstrate that thyroid hormone response element sequence plays an important role in determining the nuclear hormone receptor and coactivator requirements for TR action.

**RESULTS**

**Influence of the TRE Sequence on the Role of RXR in T3-mediated Gene Expression**—Ten naturally occurring direct repeat TREs (Table I) were inserted into yeast strains in which TRβ1, RXRα, or both together were expressed. Where indicated, incubations also included 0.2 µM of the purified TIF2 nuclear receptor interaction domain. Binding reactions were carried out at room temperature for 40 min in a total volume of 17.5 µl in binding buffer containing 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 20% glycerol, and 0.9 µg of poly(dI-dC). After incubation, the mixtures were analyzed on 6% non-denaturing acrylamide gels using 0.25× Tris borate/EDTA buffer. The gels were fixed, dried, and analyzed by autoradiography and/or PhosphorImager analysis.

**TABLE I**

| Abbreviation | Gene | Sequences | Ref. |
|--------------|------|-----------|------|
| roMHC        | Rat α-actinin heavy chain | TGGAGGTGACAGGAGGA | 6    |
| rGH          | Rat growth hormone        | GAAAGGTAAGACGACGACG | 43   |
| rP450        | Rat NADPH:cytochrome P450 oxidoreductase | GACAGTGGTACAGGACCACG | 44   |
| rME          | Rat malic enzyme          | TGGGCGTGAACGAGGACCACG | 45   |
| cME2         | Chick malic enzyme, TRE 2 | TAAAGGGCGACGACGAGCAGC | 46   |
| hDI          | Human 5′-deiodinase type 1, TRE2 | GCGGTGACGACGAGGACCACG | 47   |
| mE5          | Chick malic enzyme, TRE 5 | TGGGCGTGAACGAGGACCACG | 48   |
| rSerCa2      | Rat sarcoplasmic reticulum calcium ATPase, TRE 1 | TGGGCGTGAACGAGGACCACG | 49   |
| hPL          | Human placental lactogen  | TGGGCGTGAACGAGGACCACG | 50   |
| rUCP         | Rat uncoupling protein 1  | TAAAGGGCGACGAGGACCACG | 51   |
| 8KR          | Artificial TRE            | TGGGCGTGAACGAGGACCACG | 52   |
| 5′-Mut       | 5′R with 5′ half-site destroyed | TGGGCGTGAACGAGGACCACG | 53   |
| roMHC(TG)    | roMHC with 5′ GG to TG mutation | TGGGCGTGAACGAGGACCACG | 54   |

Production of TRβ1, RXRα, and TIF2 in E. coli — Full-length rat TRβ1, full-length mouse RXRα, and the human TIF2 nuclear receptor interaction domain (amino acids 624–869) (22) were expressed in Escherichia coli as glutathione S-transferase (GST) fusion proteins using the vector pGEX-3X (23). E. coli strain BL21 carrying the fusion protein expression vectors were grown at 30 °C. The cultures were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 2.5 h at 30 °C. To increase the solubility of the receptor fusion proteins, a plasmid expressing E. coli thioredoxin (24) was coexpressed with GST-TIF2 or GST-RXRα. After harvesting, the cells were resuspended in a buffer containing 150 mM NaCl, 10 mM sodium phosphate, pH 7.5, 2 mM EDTA, 5 mM dithiothreitol, and 1 tablet of Protease Inhibitor Mixture (Roche Molecular Biochemicals) per 25 ml, and passed through a French press twice at 1200 lb/in². The bacterial lysates were centrifuged at 10,000 × g for 15 min, and the supernatants were incubated with 1 ml of glutathione-agarose beads at 4 °C for 1 h. After washing three times with cold phosphate buffer and once with thiolating buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 2.5 mM CaCl2, 2 mM dithiothreitol, the agarose beads were resuspended in 1 ml of thiolating buffer. The TRβ1, RXRα, and TIF2 receptor interaction domains were cleaved from GST on the agarose beads by addition of 3.6 units of thrombin (Novagen, Madison, WI), incubated for 30 min at room temperature, and purified by centrifugation.

Electrophoretic Mobility Shift Assays—To perform EMSAs, double-stranded synthetic oligonucleotides were radiolabeled with [32P]dCTP by fill-in reactions using the Klenow fragment of DNA polymerase. The oligonucleotide probes used are listed in Table I. 1.5 × 10⁸ cpm of radiolabeled oligonucleotides were incubated with 0.25 µM of purified TRβ1, RXRα, or both together. Where indicated, incubations also included 0.2 µM of the purified TIF2 receptor nuclear interaction domain. Binding reactions were carried out at room temperature for 40 min in a total volume of 17.5 µl in binding buffer containing 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, 20% glycerol, and 0.9 µg of poly(dI-dC). After incubation, the mixtures were analyzed on 6% non-denaturing acrylamide gels using 0.25× Tris borate/EDTA buffer. The gels were fixed, dried, and analyzed by autoradiography and/or PhosphorImager analysis.

RESULTS

Influence of the TRE Sequence on the Role of RXR in T3-mediated Gene Expression—Ten naturally occurring direct repeat TREs (Table I) were examined in yeast. The TREs were inserted upstream of a basal cyc1 promoter linked to a β-galactosidase reporter (19) and then integrated into the chromosome of S. cerevisiae. β-Galactosidase activity assays were performed with yeast reporter strains, in which TRβ1, RXRα, or both together were expressed. Data are expressed as β-galactosidase activity relative to identical yeast strains with an empty nuclear receptor expression vector (fold induction). As shown in Fig. 1, TR mediated reporter gene induction from each of these TREs in the absence of thyroid hormone. This ligand-independent reporter gene stimulation reflects the function of the AF-1 domain of TRβ1, as it is unaffected by mutations in AF-2 (Fig. 9) or by the expression of AF-2-dependent coactivators (data not shown). The focus of these studies is not on the absolute magnitude of reporter gene induction but on the relative reporter gene induction seen in the presence of RXR + TR versus that seen in the presence of TR alone. TREs exhibit a continuous spectrum of RXR dependence, which is reflected in the ratio of β-galactosidase activity induced by RXR.
of the core hexamers, we generated a mutant rMHC TRE that influences RXR activity.

heterodimers is such that RXR occupies the 5′-T(A/G)-5′ half-site, including a T(A/G)
induction) in reporter gene activation as compared with expression of TRβ1 alone (Fig. 1, lane 1, and Fig. 2). At the other end of the spectrum, reporter gene activation from the rat uncoupling protein 1 (rUCP) TRE was almost entirely independent of RXR. TRβ1 alone activated reporter gene expression 5.3-fold, and the coexpression of RXR only resulted in a further increase of 1.2-fold (Fig. 1, lane 10, and Fig. 2).

Comparison of the TRE sequences with in vivo reporter gene activities (Fig. 2) indicates that independence of RXR for reporter gene activation correlates relatively well with the presence of the dinucleotide TG or TA upstream half-site. For example, as shown in Fig. 2, rUCP and hPL possess a TG upstream of the 5′ core hexamer and display minimal RXR dependence for TR-mediated reporter gene expression. In contrast, RXR dependence is maximal for rMHC, rGH, and rP450, none of which contains the dinucleotide T(A/G) upstream of the 5′ core hexamer. Since the polarity of RXR-TR heterodimers is such that RXR occupies the 5′ half-site (25, 26), it is reasonable that the TRE 5′ half-site sequence is the one that influences RXR activity.

To assess further the importance of the 5′ T(A/G) upstream of the core hexamers, we generated a mutant rMHC reporter construct in which the first G of the 5′ dinucleotide GG was replaced by T, creating rMHC(TG) (Table I). As predicted and shown in Fig. 3, expression of TRβ1 alone led to a 5-fold induction of β-galactosidase activity from rMHC(TG), and addition of RXR resulted in a further increase of only 1.4-fold (Fig. 3A). In contrast, expression of TRβ1 alone led to only a 1.7-fold induction from wild type rMHC, but this increased 4.3-fold with the addition of RXR (Fig. 3B). These data demonstrate that a single substitution of T for G two nucleotides 5′ to the upstream core hexamer is capable of shifting the nature of the rMHC TRE from more RXR dependence to less RXR dependence.

In summary, the results confirm that RXR is not always required for TR-mediated gene activation. The dinucleotide T(A/G) 5′ to the upstream TRE half-site is important in determining the RXR requirement for TR-mediated gene expression. However, RXR dependence is not all-or-none but, rather, varies continuously. This suggests that, in addition to the 5′ T(A/G), the sequence and spacing of the TRE half-sites may modify the RXR dependence of TR-mediated gene expression from certain TREs.

Differential Binding of TR Alone or RXR-TR Heterodimers to T3 Response Elements—To determine whether the different requirements of RXR for TR-mediated gene expression correlate with the different binding abilities of RXR-TR heterodimers versus TR alone to these TREs, we performed EMSAs using purified recombinant TRβ1 and RXRα. Fig. 4A shows that TRβ1 itself is able to bind to almost all these natural response elements, although the level of binding varies considerably. The strongest binding of TRβ1 is seen with hDI, rSerCa2, hPL, and rUCP (lanes 6, and 8–10). These TREs generally exhibit a relatively high β-galactosidase activity induced by TRβ1 alone or less RXR dependence for TR-mediated gene activation (Figs. 1 and 2). However, the binding of RXR-TR heterodimers to rSerCa2, hPL, and rUCP was essentially undetectable (Fig. 4B, lanes 8–10). In contrast, TREs such as rMHC, rP450, and rME that display more dependence on RXR for gene activation support the formation of RXR-TR heterodimers (Fig. 4B, lanes 1, 3, and 4) but bind TRβ1 alone weakly relative to rSerCa2, hPL, and rUCP (Fig. 4A, lanes 1, 3, and 4 versus 8–10). hDI binds well both to RXR-TR heterodimers and to TR alone and thus is intermediary in RXR dependence for gene activation. The correlation between RXR-TR DNA binding and RXR dependence of yeast reporter gene activity was evaluated by plotting the ratio of (RXR + TR heterodimer DNA complexes)/(TR DNA complexes) determined by EMSA versus the reporter gene induction data (Fig. 4C). This plot shows that TREs capable of forming stronger RXR-TR heterodimers are more heavily dependent on RXR for gene activation.

Since we observed that the mutant TR rMHC(TG) exhibits increased reporter gene induction by TR alone and less RXR dependence, we were interested in determining if the binding of TR to this mutant TR also is altered. EMSA showed that, as expected, the binding of TRβ1 to rMHC(TG) was greatly enhanced when compared with the binding to wild type rMHC (Fig. 4D). This indicates that a single base change in a TRE can regulate the importance of RXR for both TR binding and gene activation.

Taken together, these data suggest that the different binding affinities of TR alone versus RXR-TR heterodimers account in large part for the different requirements of RXR for TR-mediated gene expression. The importance of the 5′ dinucleotide T(A/G) in determining the RXR requirement for TR-mediated gene expression appears to be through enhancing TR binding, at least for certain TREs.

Effect of Coactivators on TR-mediated Reporter Gene Expression—Nuclear hormone receptors activate transcription in a ligand-dependent manner through an interaction of their AF-2 domains with coactivator proteins. The p160 coactivator family has previously been shown to induce hormone-dependent transcriptional activation on the artificial T3 response element 8DR4 (TAAGGTCACTCTAAAGTCA) in a yeast expression system (19). We asked whether the related p160 coactivator TIF2 (18) could potentiate hormone-dependent gene activation from these natural TREs. To do so, the previously studied yeast...
TRE Sequence and Nuclear Receptor Requirements

Fig. 2. RXR dependence of TR-mediated reporter gene expression. The ratio of β-galactosidase activity induced by RXR plus TR to TR alone was calculated from the data of Fig. 1 to reflect the RXR dependence of TR-mediated reporter gene expression. The sequences of the TRE oligonucleotides are shown without the 5'-GATC overhangs. The dinucleotide TG or TA upstream of the 5' half-site is in boldface, and the receptor binding half-sites are underlined.

Fig. 3. The effect of thymine 2 bp upstream of the 5' core hexamer on TR-mediated reporter gene expression. A, β-galactosidase activity of the rαMHC(TG) reporter construct was measured while TRβ1, RXRa, or both together were expressed in the absence of ligand. The expression of RXR alone did not induce β-galactosidase activity (data not shown). The sequence of the oligonucleotide is shown at the top of the panel. The dinucleotide TG upstream of the 5' half-site is in boldface, and the receptor binding half-sites are underlined. Results are the mean ± S.E. of four independent experiments. B, β-galactosidase assays were carried out as per A but with the wild type rαMHC reporter construct. The expression of RXR alone did not induce β-galactosidase activity (data not shown). The sequence of the oligonucleotide is shown at the top of the panel. The receptor binding half-sites are underlined. Results are the mean ± S.E. of four independent experiments.

strains were transformed with an expression vector for TIF2 (or empty vector), and β-galactosidase activity was measured following incubation with or without triac, a T3 analog.

In the absence of coactivator, β-galactosidase activity driven by all of these natural TREs was not increased by triac. Instead, triac caused a small (20%) but reproducible suppression of β-galactosidase activity (data not shown). As shown in Fig. 5, ligand induction of reporter gene expression from almost all these TREs could be restored by coexpression of TIF2 and TRβ1. However, the level of ligand-induced reporter gene expression varied considerably among the TREs. For example, β-galactosidase induction from rUCP was stimulated 6.1-fold by triac (Fig. 5, lane 10, solid bar), whereas induction from rαMHC was stimulated only 1.6-fold (lane 1, solid bar). Interestingly, coexpression of RXRα with TRβ1 and TIF2 reduced the ligand induction of reporter gene expression on nearly all TREs. For example, the triac induction from rUCP fell from 6.1- to 2.9-fold when RXR was coexpressed (lane 10). Thus, in general, TIF2 mediated ligand-dependent reporter gene expression is stronger when TR alone is present than TR and RXR.

In addition, a strong triac-dependent increase (3.4-fold) in reporter gene activation was observed from rαMHC(TG) when TIF2 and TRβ1 were coexpressed (Fig. 5, lane 11, solid bar). In the context of wild type rαMHC, such TIF2-mediated ligand-dependent activation was only 1.6-fold (Fig. 5, lane 1). This difference is consistent with the enhanced ability of rαMHC(TG) to bind TR in the absence of RXR and substantiates the preference of TIF2 as a coactivator for TR alone rather than RXR-TR.

Strong triac induction was seen only with the hDI, rUCP, and MHC(TG) TREs, all of which bind TR well in the absence of RXR. However, the rSerCa2 and hPL TREs also bind TR well, even though they support only minimal triac induction. To investigate this further, EMSAs were performed with these TREs and recombinant TR ± TIF2 (Fig. 6). Two distinct TR-TIF2-TRE complexes were identified, the slower of which correlated with triac induction in the yeast reporter gene assay. This slower complex was abolished by mutation of the 5' TRE half-site (lane 8), suggesting that it represents a TR homodimer-TIF2 complex.

The decrease in triac induction of reporter gene expression when RXR was coexpressed with TR also was investigated by EMSA. The TR-TIF2 complex with the rUCP TRE was greatly inhibited by the addition of RXR (Fig. 7, solid bar). This slower complex was abolished by mutation of the 5' TRE half-site (lane 8), suggesting that it represents a TR homodimer-TIF2 complex.

Mutational and x-ray crystallographic studies have demonstrated that the AF-2 domain of nuclear hormone receptors plays a critical role in mediating ligand-dependent transcriptional activation (5, 27). To assess whether the restoration of ligand-dependent reporter gene expression by TIF2 in these studies also is mediated through the AF-2 domain, the point mutations E457A or L454A were introduced into the TRβ1 AF-2 domain. Previous studies have shown that either E457A or L454A impairs AF-2 function in mammalian cells but does not affect the ability of the receptor to bind to DNA or to thyroid hormone (28). As shown in Fig. 8, these mutations impaired the ability of TIF2 to support triac induction of β-galactosidase activity. Although the effect of the E457A mutation was modest, L454A decreased triac induction by ~80%. This is not likely due to diminished expression, as L454A has wild type triac-independent AF-1 activity. Thus, as expected, TIF2 function is AF-2 dependent in yeast as it is in mammalian cells.

DNA SEQUENCES

CTGAGGCACTAAGGTCAAC
ATGGGGCTGAGGAGGAGG
CGGGGCGGGAGCCAGG
GTGAGGGTGAGGGAGGACT
GGGGGGTTACTCTGGGGAG
TTAGGGCGGCAGCTGGGAGC
TTGGGCTTGAGGGAGGAGC
CACAGGTGAGCTGGAGGAGC
GAAAGTTAGCTGAGGAGC
TGAGGCTGAGGGAGGAGC

3932
In the absence of coactivators such as TIF2, triac modestly represses TR-mediated reporter gene activation. Surprisingly, as shown in Fig. 9, the TR\(_{\beta 1}\) AF-2 mutations relieve this triac repression seen in the absence of TIF2. As expected, however, the TR\(_{\beta 1}\) AF-2 mutations have no effect on ligand-independent gene activation, as this is mediated through the N-terminal AF-1 domain.

**DISCUSSION**

It is widely held that, in mammalian cells, gene activation by thyroid hormone receptors is mediated via RXR-TR heterodimers (reviewed in Refs. 1, 10, and 11). This model has developed from two observations. 1) RXRs were identified as proteins that heterodimerize with and increase the DNA binding of TRs (8, 9, 29). 2) Cotransfection of mammalian cells with RXR can increase the T3 induction of reporter genes (29). However, the importance of variations in TRE sequences was not recognized when the “RXR hypothesis” was developed. In fact, the above studies utilized either the rat \(a\)-myosin heavy chain TRE (29) or inverted repeat sequences that function as promiscuous nuclear receptor response elements in transfections but that are not known to function as natural TREs \(a\) vivo (9, 30, 31).

Naturally occurring TREs generally are direct repeats (or occasionally everted repeats), and as shown in this paper, the \(a\)MHC direct repeat is more dependent on RXR for TR-DNA binding than are most other direct repeat TREs. Thus, inadvertently,
EMSAs were performed with TRβ1, T3, and 32P-labeled double-stranded probes corresponding to the TREs described in Table I, lane 10. This gel was run longer than usual to resolve the two TR-TIF2-DNA complexes, resulting in the free DNA running off the bottom of the gel. Thus, all free probe lanes demonstrated no radioactivity; this is shown for SerCa2, lane 1. The TR-DNA complexes are at the bottom of the gel, as illustrated by the arrow. This experiment was repeated twice with identical results.

Inhibition of TR-TIF2-DNA complex formation by RXR. EMSAs were performed with TRβ1, T3, and 32P-labeled rUCP TRE, the TIF2 nuclear receptor interaction domain (TIF2 amino acids 624–869) and RXR as indicated. The TR-TIF2-DNA complex is marked by the arrow. This experiment was repeated twice with identical results.

Effect of TRβ1 AF-2 mutations on TIF2-mediated ligand stimulation of reporter gene expression. β-Galactosidase activities of reporter constructs were measured while TRβ1 or TRβ1 point mutants (E457A or L454A) were expressed with TIF2 in the absence or presence of triac. Results are the mean ± S.E. of four independent experiments.

Initial investigations were performed using response element sequences that favored the role of RXR-TR heterodimers.

There is only one published report that systematically assessed the effect of cotransfected RXR on T3 inductions from several TREs in mammalian cells (32). In that publication, COS, ES, and JEG-3 cells were transfected with 7 different TRE-chloramphenicol acetyltransferase reporter plasmids along with TR and RXR. The 7 TREs are difficult to compare because they differ not only in sequence but also in the number of receptor-binding sites (2, 3, or 4) and the orientations of the binding sites. Nevertheless, certain trends were apparent. In JEG-3 cells, RXR failed to enhance gene expression from any of the TREs. In COS and ES cells, reporter gene inductions increased anywhere from nil to 3-fold, with most TREs showing small increases of 25–50%. Furthermore, stronger effects did not strictly correlate with lower endogenous RXR levels. For example, the TRE that was most affected by cotransfected RXR in COS cells (rGH TRE) showed a 3-fold increase in gene induction. However, gene expression from this TRE was not affected by exogenous RXR in ES cells, even though ES cells express less endogenous RXR than COS cells. Despite this inconsistency, the lack of effect of RXR could be interpreted to indicate that endogenous RXR is sufficient for T3 responsiveness. This would be consistent with the fact that JEG-3 cells have the most endogenous RXR of the 3 cell lines and showed no effects with exogenous RXR. However, transfection of JEG-3 cells with an RXR response element-reporter construct does not result in 9-cis-retinoic acid (the RXR ligand) induction of the reporter gene unless exogenous RXR is cotransfected (33). It is difficult to understand how cells can have enough endogenous RXR to support maximal gene inductions from TREs, yet be unable to support even minimal gene inductions from RXR response elements.

In any case, the above experiments address the function of exogenous RXR on TR function but do not really address whether RXR is fundamentally needed. This is because all mammalian cells express RXR endogenously. There are three RXR genes, denoted α, β, and γ. Mice and cell lines that are null for 5 of these 6 RXR alleles have been created, but it has not been possible to create animals or mammalian cell lines that are truly null for RXR protein. Thus, it has not been possible to determine how the absence of RXR would influence TR-dependent gene expression in mammalian cells.

It is for this reason that we used S. cerevisiae as a model system for our experiments. Since yeast lack nuclear receptors (and coactivators), they represent a null system into which TR, RXR, and coactivators can be added or not, thus allowing a true test of the consequences of RXR deficiency. Although it is appropriate to interpret results in S. cerevisiae with caution,
the track record of yeast as a model system for nuclear receptor action has been impressive. Several laboratories have shown that TRs and other nuclear hormone receptors can function as ligand-independent or -dependent transcription factors in *S. cerevisiae* (19, 34–37). The essential role of SWI/SNF proteins in nuclear receptor action was first demonstrated in an *S. cerevisiae* model system (38) and was subsequently confirmed in mammalian cells (39). Similarly, the role of Ada proteins in glucocorticoid receptor action was first demonstrated in *S. cerevisiae* (40). In both of these cases, the yeast model system was uniquely valuable because yeast cells existed that were null for the SWI/SNF or Ada proteins when equivalent mammalian systems did not exist. We have chosen to utilize a yeast model system to study the role of RXR in TR function for analogous reasons.

As many as 8% of genes expressed in liver may be T₃-responsive (41), but only a small number of TREs have been well characterized. We selected 10 naturally occurring direct repeat response elements for study. These TREs vary considerably in sequence, but all have two directly repeated half-sites. Thus, by comparing the sequences of the TREs with their activities in yeast, we may decipher the sequence features for RXR-independent gene expression from direct repeat response elements without the complexities caused by variations in half-site orientation and number.

In mammalian cells, the unliganded TR generally functions as a transcriptional repressor, which is mediated through interaction with a co-repressor complex. In yeast, the corepressors that interact with TR are absent, thus allowing TR to display ligand-independent transcriptional activity (2, 19, 37). This study demonstrates that TR-mediated reporter gene stimulation from natural TREs is differentially enhanced by RXR in the absence of ligand. These TREs exhibit a continuous spectrum of RXR dependence. Comparison of the TRE sequences with *in vivo* gene expression patterns suggests that independence of RXR generally is associated with the presence of the 5′ dinucleotide TG or TA upstream of the 5′ half-site. In addition, the importance of the 5′ T(A/G) was confirmed by creating a mutant reporter construct roMHC(TG) that is identical to the wild type roMHC except the dinucleotide TG replaces the wild type GG upstream of the 5′ half-site. In contrast to wild type roMHC, roMHC(TG) is largely RXR-independent.

If the 5′ T(A/G) were the sole determinant of RXR independence, TREs would fall into two discrete groups, complete independence of RXR or complete dependence. This, however, is not what was observed. Rather, the importance of RXR varies in a continuous fashion from near total dependence to near total independence. This indicates that there must be modifying influences beyond the presence or absence of the 5′ T(A/G). Of the 10 response elements in Fig. 2, the 5 at the extremes all follow the T(A/G) rule (the three TREs with the greatest RXR dependence all lack T(A/G), and the two TREs with the greatest RXR independence contain T(A/G)). The TREs in the middle may or may not contain the 5′ T(A/G), suggesting that minor differences in TRE sequence can modify the influence of the T(A/G), thus moving the TRE from one of the extremes toward the middle of the spectrum of RXR dependence. A comparison of the 10 TRE sequences does not reveal any clear rules as to what these modifying influences may be, and it is likely that many sequence differences each may contribute a little.

*In vitro* DNA binding assays revealed that TR itself is able to bind to almost all these natural response elements. Functional independence of RXR generally correlates with the strong binding of TR alone to the TREs. Importantly, we also show that the conversion of the roMHC TRE from more RXR dependence to less RXR dependence (roMHC(TG)) is due to the increase in TR binding to DNA. The hDI TRE is unusual in that it binds well to TR alone and to RXR-TR heterodimers, which explains why its RXR dependence is intermediate. The relationship between receptor-DNA binding and reporter gene activation for the hDI TRE is similar to that of the other TREs (Fig. 4C). However, for all TREs, a perfect quantitative relationship between receptor-DNA binding and reporter gene activation does not exist, suggesting that DNA binding may not be sufficient to lead to transcriptional activation of the target genes.

In mammalian cells, TR functions as a ligand-activated transcription factor, TR modulation of gene expression involves the coordination of an array of coregulatory proteins including coactivators and corepressors. In the absence of ligand, TR interacts with a corepressor complex and inhibits positively regulated gene transcription. The binding of ligand triggers a conformational change in the TR that results in replacement of the corepressor complex by a coactivator complex, thereby leading to activation of transcription. In the yeast model system, addition of thyroid hormone is unable to elevate reporter gene expression above the level achieved by the ligand-independent activity of TR (AF-1 activity), due to the lack of AF-2-dependent nuclear receptor coactivators. As expected, however, coexpression of the coactivator TIF2 in yeast can restore ligand-dependent gene activation from nearly all these TREs.

Although three of the TREs supported triac inductions of 4-fold or more, most showed inductions of 2-fold or less (Fig. 5). This low level of hormone induction is not surprising, as thyroid hormone has very modest effects on endogenous gene expression in mammalian cells. For example, a cDNA microarray analysis identified 14 hepatic genes induced by T₃ *in vivo*; of these, only one was induced more than 4-fold and 8 were induced less than 3-fold (42). Furthermore, in mammalian cells T₃ induction is a consequence of both AF-1 and AF-2 activities. However, as noted above, yeast lack corepressors, resulting in constitutive AF-1 activity. Thus, in yeast, hormone induction reflects only AF-2 activity.

Surprisingly, we observed that the effect of TIF2 on ligand-dependent activity is more potent in the presence of TR alone than RXR plus TR, regardless of the nature of the TRE. The observation that TIF2-mediated ligand-dependent activity is markedly increased in the context of roMHC(TG) relative to wild type roMHC is consistent with this, because the ability of roMHC(TG) to bind TR alone is much stronger than that of wild type roMHC. Gel shift studies with the rUCP TRE demonstrated that RXR inhibits formation of the TR-TIF2-DNA complex, consistent with the yeast reporter gene data.

Overall, the data suggest that TRE sequence variations influence the roles of RXR and specific coactivators in TR-mediated gene expression. The availability of RXR and/or specific coactivators thus could differentially regulate the T3 induction of subsets of hormonally responsive genes within a cell. It will be important to develop mammalian model systems to test these principles.

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