5'-Flanking Sequences in Thyroid Hormone Response Element Half-sites Determine the Requirement of Retinoid X Receptor for Receptor-mediated Gene Expression*

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David P. Olson and Ronald J. Koenig‡

From the Division of Endocrinology and Metabolism, University of Michigan Medical Center, Ann Arbor, Michigan 48109-0678

Thyroid hormone receptors are ligand-inducible transcription factors that can potentially interact with thyroid hormone response elements as homodimers or heterodimers with the retinoid X receptor. It has generally been felt, however, that the heterodimer is responsible for induction of gene expression. We have demonstrated previously that the optimal thyroid hormone receptor binding sequence is not the consensus hexamer half-site AGGTCA but is an octamer, TAAGGTCA. Based upon these findings, we hypothesize that thyroid hormone response elements composed of optimal half-sites (TAAGGTCA) will bind thyroid hormone receptors readily and activate gene expression independently of the retinoid X receptor. In contrast, response elements composed of suboptimal half-sites (e.g. GCAGGTCA) will require the retinoid X receptor to facilitate thyroid hormone receptor-mediated gene expression. To test this hypothesis, we have reconstituted thyroid hormone receptor-mediated gene expression in yeast. Our studies confirm the hypothesis that the retinoid X receptor is required for gene expression from response elements composed of suboptimal half-sites, whereas thyroid hormone receptors are sufficient to activate gene expression maximally from response elements containing optimal half-sites. Furthermore, coexpression of steroid receptor coactivator-1 is required for ligand-dependent gene activation from single response elements. Surprisingly, however, coexpression of the retinoid X receptor decreases the steroid receptor coactivator-1-dependent thyroid hormone induction. Overall these data demonstrate that the architecture of the thyroid hormone response element dictates the nuclear receptor requirements for gene activation. The studies suggest that different coactivators may be required for gene activation depending upon the response element architecture and the nature of the bound thyroid hormone receptor complex (homo- versus heterodimer).

Thyroid hormone receptors (TRs) are zinc finger transcription factors in the erbA superfamily that bind DNA at specific response element sequences (thyroid hormone response elements, TREs) and activate gene expression in response to thyroid hormone (T3) (for reviews, see Refs. 1 and 2). TRs have been shown to bind DNA as monomers, homodimers, or heterodimers with another erbA superfamily member, the retinoid X receptor (RXR) (3–5). Heterodimerization of TR with RXR profoundly augments the association of TR with certain TREs in vitro (3–5), yet over-expression of RXR in mammalian cells only modestly (or sometimes not at all) enhances the T3 responsiveness of downstream genes (6). This discrepancy has confounded the assignment of a physiologic role for RXR in vivo.

TREs generally are composed of hexameric half-sites oriented as direct repeats spaced by four base pairs, palindromes separated by 0–1 base pairs or inverted palindromes spaced by six base pairs (7–11). TR-mediated gene activation varies with half-site orientation and spacing and can be modulated by sequence variations within and immediately flanking the half-sites (12, 13). Moreover, the conformation of the TR-RXR heterodimer changes when the heterodimer binds different DNA elements (14). Such protein conformation differences have been correlated with the altered transcriptional activities of various TREs, further indicating the importance of response element architecture in modulating hormonal responsiveness.

Systematic mutational analysis of the rat growth hormone promoter TR led to the proposal of the hexamer AGGTCA as the consensus half-site for high affinity TR binding (7). Using a non-biased polymerase chain reaction selection strategy, we have previously shown that the TR monomer optimally binds the octamer TAAGGTCA rather than the proposed hexameric consensus sequence (15). Based on these findings, we hypothesize that sequences flanking the hexameric half-sites which compose the TRE will dictate the requirement for RXR in mediating thyroid hormone responsiveness. Specifically, TREs composed of optimal half-sites will not require RXR to activate gene expression, since TRs should associate readily with these high affinity sites. TREs composed of suboptimal half-sites (such as GCAGGTCA) will depend on RXR to facilitate TR interactions with these half-sites and modulate transcriptional responses to thyroid hormone.

The ubiquitous expression of RXR in mammalian cells complicates the interpretation of studies designed to test the above hypothesis. To circumvent the problem of endogenous RXR expression, we have reconstituted a model system in the lower eukaryote, Saccharomyces cerevisiae, to mimic mammalian nuclear receptor gene activation. S. cerevisiae has been used to model gene activation by several nuclear hormone superfamily members, including TRs, retinoic acid receptors, vitamin D receptors, estrogen receptors, and glucocorticoid receptors (16–22). S. cerevisiae lacks known homologs of nuclear receptors but has sufficient homology with higher eukaryotes in its basal
transcription machinery to support nuclear receptor-mediated transcriptional activation. Given this null receptor background, one can express different combinations of nuclear receptors in the context of defined TREs and determine the receptor requirements dictated by the TRE for TR-mediated gene expression in vivo.

Unlike glucocorticoid receptors that translocate to the nucleus only after binding the appropriate hormone, TRs are constitutively found in the nucleus, bound to TREs even in the absence of ligand (23). In mammalian cells, TRs are able to activate or repress the transcription of downstream genes in promoter-specific contexts (for review, see Ref. 24). Recent studies have identified several auxiliary proteins that associate with nuclear receptors and function physiologically to affect hormone responsiveness. In the absence of T3, nuclear corepressors (such as the nuclear receptor corepressor-25 or the silencing mediator for retinoid and thyroid hormone receptors (26)) associate with TRs and repress gene expression. T3 binding produces a conformational change in the TR (27) that dissociates the corepressor and recruits coactivator proteins (such as the steroid receptor coactivator-1, SRC-1 (28), or the CREB binding protein (29)) that stimulate transcription presumably through interaction with the basal transcription machinery. Little is known regarding the impact of response element architecture on coactivator function. Expression of coactivators in the reconstituted yeast nuclear hormone receptor model can help clarify the role of TRE structural variations in the molecular mechanisms of coactivation.

The studies reported demonstrate that, as hypothesized, TREs composed of suboptimal TR half-sites require RXR for TR-mediated gene expression, whereas TREs composed of optimal TR half-sites do not. In addition, the presence of the coactivator protein SRC-1 is required to confer ligand dependence to TR-mediated gene activation from single copy response elements. Interestingly, the 5′-dinitro-flanking the consensus hexamer half-site appears to be a determinant of coactivator function as well.

EXPERIMENTAL PROCEDURES

Construction of Reporter and Expression Plasmids—An integrating reporter plasmid 83:305 was generated by ligating the SalI-Sclal fragment of pCM83 (30), containing the CYC1 promoter fused to the β-galactosidase gene, into pRS305 (31) at the SalI, NgoMI (filled in) sites. Oligonucleotide response elements with GATC overhangs were synthesized (see Table I) and inserted into the BglII site upstream of the CYC1 promoter. All response element reporter constructs were sequenced (see below). Protein extracts were prepared by glass bead disruption in the yeast genome. Rat TRβ1 (32) and mouse RXRs (5) were subcloned into the pRS-GalH expression vector2 on opposite sides of the bi-directional, galactose-inducible promoter to allow expression of both receptors from a single plasmid. A SalI-SmaI fragment encoding SRC-1 (28) was subcloned into the yeast expression vector p413-TEF (33) using the SalI-Xhol (filled in) restriction sites.

Transformation of S. cerevisiae with Integrating Reporter and Expression Vectors—Transformation of S. cerevisiae strain SEY6210 (MATα; ura3-52; leu2-3,112; his3-D200; trp1-A901; lys2-801; suc2-A9) with each of the integrating reporter constructs shown in Table I was carried out using a standard lithium acetate protocol (34). 25–50 μg of each 83:305 reporter plasmid was linearized within the LEU2 locus by digestion with BstEII prior to transformation. Transformation of yeast with episomal expression vectors was performed with 5 μg of nonlinearized plasmid. Following transformation, cultures were plated on appropriate synthetic selection media, and single colonies were isolated. Southern analysis was performed to determine the number of reporter constructs integrated by homologous recombination in the leu2 insertion site (data not shown). Strains bearing single reporter construct insertions were selected for further study.

Induction of Reporter Gene Expression—Prior to exposure to hormone, yeast strains were grown in appropriate selection medium containing 3% glycerol and 3% ethanol, a non-repressing carbon source that does not induce nuclear receptor expression from the galactose promoter. Cultures were diluted to an absorbance of 0.2 at 650 nm (A650) and grown overnight (12–16 h) at 30 °C, 300 rpm in the presence of 3% β-galactose (to induce nuclear receptor expression) with or without 1 μM triac (3,5,3′-triiodothyroacetic acid), a T3 analog.

Determination of β-Galactosidase Activity—Following overnight exposure to hormone, the A650 of each culture was measured as an indicator of culture density. 1-ml aliquots of each culture were pelleted by microcentrifugation; the supernatants were removed and the pellets stored at −20 °C. Frozen cell pellets were thawed, resuspended in 700 μl of 2 buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgCl2, 0.1% β-mercaptoethanol), and solubilized with 2 μg/ml of chloroform and 0.1% SDS. Pellets were vortexed briefly and incubated at 30 °C for 5 min. 200 μl of the substrate o-nitrophenyl-β-D-galactopyranoside (4 mg/ml 0.1 mM NaPO4, pH 7.0) was added, and the reactions were incubated at 30 °C. Upon appearance of yellow color, 350 μl of 1 M Na2CO3 was added to stop the reaction, and the time was noted. Samples were then microcentrifuged to remove debris, and the absorbance of the supernatant at 420 nm (A420) was determined. β-Galactosidase activity was calculated as follows in Equation 1:

\[
\text{A}_{420} \times \text{volume of cells pelleted (ml)} \times \text{reaction time (min)} \times 1000
\]

In preliminary experiments the assay was shown to be linear with respect to cell protein and reaction time. β-Galactosidase activity was normalized to the level of activity measured in each reporter strain transformed with an empty pRS-GalH expression vector.

Semi-quantitative Western Blot Analysis of Nuclear Receptors in Yeast—The TRβ1 and mouse RXRs were transfected from the vector pBluescript and then translated in vitro in the presence of [3H]leucine using Promoza transcription/translation reagents. Titrated cpm of tri-iodoacetic acid-precipitable protein were determined, and radiolabel incorporation into protein of appropriate size was confirmed by SDS-polyacrylamide gel electrophoresis and autoradiography (data not shown). After adjusting for the moles of leucines per mol of TR and RXR, these materials were used in a series of increasing doses as standards in a Western blot of yeast-expressed TR and TX. Yeast strains bearing an integrated SDR4 reporter construct and pRS-GalH and p413-TEF expression vectors were grown under inducing conditions (see above). Protein extracts were prepared by glass bead disruption in 20 mM Tris, pH 8.0, 10 mM MgCl2, 1 mM EDTA, 10% (v/v) glycerol, 2 mM dithiothreitol, 0.4 M KCl, 0.3 M (NH4)2SO4, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin, and 2 μM Leupeptin (35). Protein concentration was then determined using the Bio-Rad Protein Assay. Following SDS-polyacrylamide gel electrophoresis, 3H-proteins and yeast extracts were transferred to nitrocellulose using 60 V for 20 min in a Hoeffer TransPhor apparatus. Nitrocellulose-bound proteins were probed with anti-TRβ1 (TRβ1-352, Santa Cruz Biotech) or anti-RXRα (IIX-612) monoclonal antibodies, and antigen-antibody complexes were then visualized using an enhanced chemiluminescence detection system (Amersham Corp.).

Electrophoretic Mobility Shift Assay of TR-TR Homodimer Binding—Electrophoretic mobility shift assays were performed essentially as described (12) using 30,000 cpm of [3H]labeled double-stranded oligonucleotide and 2 μl of in vitro translated rat TRβ1 or mouse RXRα (1RX-6G12 monoclonal antibodies, and antigen-antibody complexes were then visualized using an enhanced chemiluminescence detection system (Amersham Corp.).

Expression of Nuclear Receptors in S. cerevisiae—Western blot analysis of yeast protein extracts was performed to demonstrate that TRβ1 and RXRs were expressed appropriately and at roughly equivalent levels from the bi-directional expression vector pRS-GalH. Increasing doses of in vitro translated [3H]-TRβ1 and [3H]-RXRs were included in the assay as standards for both size and mol equivalent and were run on a 15% polyacrylamide gel (Fig. 1). Control yeast protein extracts from strains expressing just TRβ1 (lane 7) or just RXRα (lane 15) showed no cross-reactivity with the anti-RXR antibody or the anti-TRβ1 antibody, respectively. Comparison of lanes 17–18 with lanes 11–13 and lanes 8–9 with lanes 2–5, as well as a repeat experiment not shown,

2 D. Thiele, personal communication.
indicates that TRβ1 and RXRα are expressed at equivalent levels following an overnight galactose induction.

Functional Reconstitution of TR-mediated Gene Expression in Yeast—To test the yeast model system for functional reconstitution of thyroid hormone responsiveness, β-galactosidase induction assays were performed with a yeast strain bearing a reporter construct with three optimal direct repeat response elements oriented head to tail upstream of the β-galactosidase reporter gene (3x8DR4). Expression of TRβ1 alone in the context of this triple optimal element mediated a 2.0-fold induction of reporter expression in the absence of ligand (Fig. 2). Addition of triac resulted in a 7.3-fold induction, which is a 3.6-fold increase over the induction achieved in the absence of ligand. Coexpression of RXRα with TRβ1 slightly increased the ligand-independent induction to 3.0-fold. With the addition of ligand, reporter gene expression was induced 5.0-fold over basal, yielding a modest 1.7-fold ligand-dependent induction. Thus, the triple optimal TRE (3x8DR4) was able to support both ligand-independent and ligand-dependent gene activation in the presence of TRβ1 alone and TRβ1 with RXRα. Other groups have made similar observations regarding TR-mediated ligand-independent (36) and ligand-dependent (16, 17) gene activation in yeast model systems, although RXR was shown to modestly augment T3 responsiveness in the context of two copies of a palindromic TRE positioned upstream of a basal promoter. The ability of RXR to augment T3 responsiveness from this palindrome element but slightly inhibit responsiveness from the triple optimal direct repeat element could relate to the differences in nucleotide sequence flanking the half-sites in each TRE or the half-site orientations.

Reporter Gene Expression from Direct Repeat, Palindromic, and Inverted Palindromic Response Elements—The juxtaposition of multiple response elements within the triple element TRE may create different TREs across the junctions of single elements and complicate the interpretation of the β-galactosidase induction results. To avoid this complication, reporter constructs with just single copy response elements were used for subsequent experiments. To investigate the importance of TRE half-site sequence and orientation in modulating gene expression, we have constructed a series of different response elements, each comprising a half-site sequence and orientation in modulating gene expression. Optimal direct repeat or palindromic TREs, however, are able to activate gene expression in the context of TRβ1 alone, suggesting the importance of TR-TR homodimers in directing gene expression from this subset of TREs. Moreover, as our hypothesis would predict, coexpression of RXRα with TRβ1 fails to activate gene expression from the optimal ligand.

Coexpression of TRβ1 with RXRα, however, resulted in a 4-fold induction of reporter gene expression in the absence of ligand. Addition of 1 μM triac slightly reduced the level of activated gene expression to 3-fold over basal. In contrast to the results with 6DR4, if the response element was composed of optimal TR half-sites (8DR4), TRβ1 alone was sufficient to induce a 5-fold increase in reporter gene expression that was independent of triac addition (Fig. 3A, right). Coexpression of RXRα with TRβ1 did not substantially augment this gene activation. As seen with the suboptimal 6DR4, ligand slightly decreased the receptor-dependent induction. As expected, expression of RXRα alone did not induce reporter gene expression above basal levels from either TRE. Thus, the optimal direct repeat TRE (8DR4) can be activated by TRβ1 alone, and coexpression of RXRα with TRβ1 has little effect on this level of activation. In contrast, the suboptimal direct repeat (6DR4) requires RXRα with TRβ1 to mediate reporter gene expression. It is noteworthy that the level of reporter gene induction is similar on 6DR4 and 8DR4; what differs is the necessity (or lack thereof) of RXR to achieve this induction. These data support the hypothesis that the affinity of the TRE for TR dictates the requirement for RXR in modulating gene expression. In addition, the ligand-dependent induction from the triple optimal direct repeat (3x8DR4) appears to be a consequence of response element multimerization, since induction from the single TREs is slightly inhibited by addition of triac.

Palindromic arrangements of suboptimal and optimal TR half-sites responded qualitatively similarly to the direct repeats, although in general the levels of induction are less. Thus, reporter gene expression from the suboptimal palindrome (6PAL0) was dependent upon coexpression of RXRα with TRβ1, and the addition of triac had little effect (Fig. 3B, left). In contrast, the optimal palindrome (8PAL0) was activated in the presence of TRβ1 alone, and coexpression of RXRα with TRβ1 had little effect on these levels (Fig. 3B, right). Interestingly the suboptimal or optimal inverted palindromes (6IP6 or 8IP6) were unable to direct reporter expression above the basal level with any combination of nuclear receptors (Fig. 3C).

We conclude from these studies that suboptimal direct repeat or palindromic TREs require RXRα with TRβ1 to induce gene expression. Optimal direct repeat or palindromic TREs, however, are able to activate gene expression in the context of TRβ1 alone, suggesting the importance of TR-TR homodimers in directing gene expression from this subset of TREs.
TRE Structure Determines RXR Requirements

The top strands of the TRE oligonucleotides are shown without the 5'-GATC overhangs. The 6 or 8 preceding the orientation designations (DR, direct repeat; PAL, palindrome; IP, inverted palindrome) indicates a suboptimal hexameric (AGGTCA) or optimal octameric (TAAGGTCA) half-site, respectively. The consensus hexamer is in boldface, and the 5'-dinucleotide TA is underlined in each optimal half-site. The number following the orientation designation (4, 6, 8, or 9) indicates the number of nucleotides separating the consensus hexameric half-sites.

| Response elements | DNA sequence |
|-------------------|--------------|
| 6DR4 (suboptimal direct repeat) | AGCAGGTCAATAGGTCACG |
| 8DR4 (optimal direct repeat) | CTAAGGTCACTTAGGTCAC |
| 6PAL0 (suboptimal palindrome) | CGGACGGCATGACCTGGG |
| 8PAL0 (optimal palindrome) | CTAAGGTCACTAGGTCAC |
| 6IP6 (suboptimal inverted palindrome) | GTGACCTATAGGTCAC |
| 8IP6 (optimal inverted palindrome) | GTGACCTACTAGGTCAC |
| 8DR9 | TTAGGTCACGATTCGTAAGGTCAC |

### FIG. 3. β-Galactosidase inductions from single direct repeat, palindromic, or inverted palindromic TREs

A. inductions of yeast strains harboring the single suboptimal (6DR4) or optimal (8DR4) direct repeat reporter construct. B, inductions from the suboptimal (6PAL0) or optimal (8PAL0) palindrome reporter constructs. C, inductions from the suboptimal (6IP6) or optimal (8IP6) inverted palindrome reporter constructs. Data represent the means of four independent experiments ± standard error (except 6IP6 + RXR – triac, n = 3). Solid bars represent inductions in the absence of triac; hatched bars represent inductions in the presence of 1 μM triac (final). Data are represented as fold inductions over the level of reporter gene expression found in the indicated yeast strain bearing an empty nuclear receptor expression vector.

TREs above the levels attained in the presence of TRβ1 alone.

To assess whether the optimal TRE requires the formation of a TR-TR homodimer or just the independent association of two TRβ1 monomers to activate gene expression, we constructed a yeast strain with a direct repeat TRE containing two optimal half-sites spaced by nine nucleotides (8DR9, see Table I). This arrangement moves the center of the upstream half-site one-half helical turn away from the downstream site and should preclude homodimer formation. This increased separation of the optimal half-sites effectively reduced the level of TRβ1-mediated reporter gene expression from 5-fold (Fig. 3A) to 1.48 ± 0.06-fold (mean ± S.E.; n = 4). Coexpression of RXRα with TRβ1 had no effect on this low level of induction (1.50 ± 0.05-fold; n = 4). Therefore, the ligand-independent activation seen from optimal TREs appears to be dependent upon TR-TR homodimer formation.

**Effect of Ligand on TR-TR Homodimer Formation**—It might be considered surprising that TR-TR homodimers could be active in yeast, especially in the presence of ligand, since T3 has been shown in vitro to destabilize TR-TR homodimers (but not the TR-RXR heterodimers) bound to direct repeats and inverted palindromes of the consensus hexamer (37, 38). To explore this issue, we have used electrophoretic mobility shift assays to examine the effect of ligand on TR-TR homodimers bound to our direct repeats (6DR4 and 8DR4) and inverted palindromes (6IP6 and 8IP6). As expected, TR-TR homodimers formed more readily on the optimal than on the suboptimal TREs (Fig. 4). PhosphorImager analysis indicated that in the absence of ligand, TR-TR homodimer formation was 15 times greater on 8DR4 than on 6DR4. The addition of T3 disrupted TR homodimer formation on the suboptimal element 6DR4 but just 40% of TR-TR homodimer formation on the optimal direct repeat 8DR4. Thus, even in the presence of ligand, TR-TR binding to the optimal direct repeat 8DR4 remained substantial and was 70-fold greater than the homodimer binding observed on the suboptimal direct repeat 6DR4. The suboptimal and optimal inverted palindromes, 6IP6 and 8IP6, gave similar results, although the effect of T3 on homodimer binding to 8IP6 was even more modest than that seen with 8DR4. These data, in combination with the yeast induction assays using 6DR4 and 8DR4, support the functional importance of TR-TR homodimers in vivo.

**Coexpression of the Coactivator SRC-1 in the Context of Direct Repeat TREs**—A current model of ligand-inducible nuclear receptor gene activation proposes that the binding of ligand by the thyroid hormone receptor results in a conformational change leading to the recruitment of coactivator proteins to the receptor dimer-DNA complex with subsequent activation of the basal transcription machinery (25). SRC-1, steroid receptor coactivator-1, has been shown (to enhance thyroid hormone responsiveness in a mammalian cell culture system (28). We sought to investigate whether expression of SRC-1 in yeast could restore thyroid hormone responsiveness from the single copy TREs. To that end SRC-1 was expressed in each of the yeast strains described above, nuclear receptor expression was induced, and β-galactosidase activity was measured following
incubation with or without triac.

SRC-1 expression had essentially no effect on reporter gene activation from the suboptimal TRE 6DR4 (Fig. 5, left; compare with Fig. 3A, left). Thus, SRC-1 did not alter the ligand-independent response to TR with or without RXR and, contrary to expectations, did not restore a ligand-dependent induction. In contrast to the results with 6DR4, however, expression of SRC-1 with the nuclear receptors in the context of the optimal direct repeat (8DR4) had profound effects (Fig. 5, right; compare with Fig. 3A, right). In the absence of ligand, SRC-1 slightly increased the levels of reporter gene expression to 5.5-fold from 5.0-fold with TRβ1 alone and to 7.0-fold from 6.1-fold with TRβ1 and RXRα together. More importantly, upon the addition of triac, TRβ1 and SRC-1 together directed a 15.1-fold induction of reporter gene expression, which represents approximately three times the level of induction seen in the absence of ligand. Unexpectedly, coexpression of RXRα with TRβ1 and SRC-1 reduced the reporter gene induction in the presence of ligand to 10.6-fold, which represents just a 1.5-fold ligand-dependent effect. In summary, expression of SRC-1 restored ligand-dependent gene activation from the optimal direct repeat TRE (8DR4) in the presence of TR alone and, to a lesser extent, in the presence of TR plus RXR. The suboptimal direct repeat TRE (6DR4), however, did not support ligand-dependent activation of gene expression even in the presence of SRC-1.

To assess the importance of TR-TR homodimer formation (as opposed to TR monomers) in modulating the SRC-1-dependent ligand induction from 8DR4, we coexpressed TRβ1 and SRC-1 in a yeast strain bearing the 8DR9 response element, induced expression of the nuclear receptors in the presence or absence of ligand, and assayed for reporter gene expression. SRC-1 did not alter the low levels of ligand-independent gene expression found in the 8DR9 strains expressing TRβ1 alone (1.43 ± 0.07 fold; n = 4). Addition of ligand resulted in a minimal increase in reporter gene expression with TRβ1 and SRC-1, from 1.43-fold over basal to 2.04 ± 0.09-fold (n = 4). This was 7-fold less than the SRC-1 and ligand-dependent induction from 6DR4 (Fig. 5, right). Coexpression of RXRα with TR and SRC-1 had little effect on reporter gene expression in the absence (2.08 ± 0.27-fold; n = 4) or presence (2.04 ± 0.17-fold; n = 4) of ligand. The data show that separation of the optimal TR half-sites by 5 base pairs drastically impaired the ability of SRC-1 to enhance gene expression in response to ligand. Therefore, in this system, SRC-1 appears to activate gene expression through liganded TR-TR homodimers and not TR monomers.

**SRC-1 Activity with Suboptimal TREs Is Dependent upon Half-site Orientation**—As in the case with the suboptimal direct repeat TRE 6DR4, coexpression of SRC-1 with TRβ1 alone in strains bearing suboptimal palindromic (6PAL0) or inverted palindromic (6IP6) TREs had no effect on gene activation in the presence or absence of triac (Fig. 6, left panel; compare with Fig. 3, B left and C left). In the absence of ligand, coexpression of SRC-1 with TR and RXR also had no effect on reporter gene expression from any suboptimal TRE. Interestingly, however, SRC-1 expression with TR and RXR did restore a modest level of ligand-dependent gene activation in the context of 6PAL0, but not 6IP6 or, as shown above, 6DR4 (Fig. 6, right panel). Therefore, both the palindromic half-site orientation and the presence of RXR with SRC-1 and TR were requirements for ligand-dependent gene activation from suboptimal TREs.

**SRC-1 Demonstrates Activity in the Context of All Optimal TREs but Is Less Effective in the Presence of RXR**—As seen with the optimal direct repeat 8DR4, expression of SRC-1 in the context of the optimal palindromic and inverted palindromic TREs had little effect on reporter gene expression in the absence of ligand (Fig. 7; compare with Fig. 3, B and C). Also as seen with 8DR4, ligand-dependent gene activation from 8PAL0 and 8IP6 was restored after coexpression of SRC-1 with TRβ1 alone and with TRβ1 and RXRα together (Fig. 7). Coexpressing SRC-1 with TRβ1 in the context of the optimal palindromic TRE (8PAL0) resulted in a 2.1-fold induction of reporter gene expression in the absence of ligand, which increased to 6.6-fold with the addition of triac. This ligand-dependent 3-fold increase in reporter gene expression was equivalent to the increase seen with TRβ1 and SRC-1 coexpression in the optimal direct repeat (8DR4) strain. SRC-1 and TRβ1 also were able to mediate a ligand-dependent 2-fold increase in reporter expression from the optimal inverted palindromic (8IP6). It should be noted, however, that the overall level of gene induction was greatest for 8DR4 and least for 8IP6; ligand-dependent fold inductions were approximately equal due to differences in the levels of reporter gene inductions by unliganded receptor.

The addition of RXRα to TRβ1 and SRC-1 attenuated the magnitude of the ligand-dependent induction in yeast strains carrying the optimal direct repeat or palindromic TREs (Fig. 7, right versus left). To determine if its effects on SRC-1-dependent ligand induction from 8DR4 (noted in Fig. 5), coexpression of RXRα with TRβ1 and SRC-1 reduced the ligand- and SRC-1-dependent induction from 3- to 1.8-fold from the optimal palindromic TRE 8PAL0. Thus, whereas the coexpression of RXR with TR and SRC-1 was required for ligand-dependent induction from the suboptimal palindromic TRE (8PAL0), RXR diminished the ability of SRC-1 to support triac-induced gene expression from the optimal direct repeat (8DR4) and palin-
TRE Structure Determines RXR Requirements

The modest augmentation of T3-responsive gene induction by RXR over-expression in cultured mammalian cells is discordant with the ability of RXR to dramatically enhance the association of TRs with certain TREs in vitro. Recently, Hsu et al. (6) examined the importance of RXR in modulating T3-dependent gene expression from several naturally occurring TREs in different cell lines and found that RXR augmentation of T3 response was TRE- and cell line-specific. This effect was quite modest (2-fold or less) and was most pronounced with more complex TREs in cell lines expressing low levels of endogenous RXR. Such cell culture studies designed to address the physiological importance of RXR in modulating T3-dependent gene activation are complicated by the ubiquitous expression of RXR in mammalian cells. The ideal system for investigating the functional role of RXR in T3-mediated gene expression is an RXR-null eukaryotic background into which RXR can be expressed. However, we have analyzed this cell line by Western blot and electrophoretic shift mobility assay and by both assays found it to contain RXR (data not shown). Thus, currently there is no RXR-negative mammalian cell line available for study. Therefore, to avoid the complications of endogenous RXR activity, we selected S. cerevisiae as the RXR-null eukaryotic background for our studies. S. cerevisiae maintains enough functional homology with mammalian transcription systems to allow modeling of mammalian nuclear hormone-mediated gene activation and has been a useful tool for understanding the molecular mechanisms by which several members of the nuclear hormone receptor superfamilies mediate gene expression in response to hormone ligands (16–22). In the absence of an RXR-null mammalian cell line, S. cerevisiae provides an excellent system for studying mechanisms of T3-dependent gene expression.

Based upon our previous identification of the optimal TR-homodimer half-site (15), we hypothesized that simple TREs composed of optimal binding half-sites would not require RXR for efficient gene activation, since TRs should bind tightly to these elements. In contrast, TREs composed of suboptimal TR binding half-sites would depend upon RXR to facilitate TR interaction with the suboptimal TRE and bring about gene activation. We find that this is true for direct repeat and palindromic TREs with regard to ligand-independent receptor-mediated activation (Fig. 3, A and B). RXR is required for gene expression from the suboptimal versions of these TREs. In contrast, TR alone activates gene expression from the optimal versions of these TREs, and this activation is not enhanced by coexpression of RXR. Furthermore, this TR-mediated activation appears to require the formation of TR–RXR homodimers rather than the association of monomeric TRs with the TRE.

Even though the optimal half-site is not found in any of the currently characterized naturally occurring TREs, it remains plausible that TR–RXR homodimers could activate a subset of optimal or near-optimal TREs. As many as 8% of liver genes may be T3-responsive (39) but only approximately a dozen natural TREs have been characterized. We postulate that the promoters of thyroid hormone-regulated genes exhibit a spectrum of RXR dependence in mediating responses to T3. At one end of this spectrum would be TREs that could support T3-dependent gene activation independent of RXR; at the other extreme would be TREs that demonstrate total dependence on RXR for mediating T3 responses. The optimal and suboptimal TREs in this study were selected to represent the ends of such a spectrum, but most natural TREs would likely fall somewhere in between. It is difficult to predict, based simply on inspection, where the currently known TREs would lie in this spectrum. These natural TREs vary not only in half-site sequence but also in half-site orientation and number, and each of these factors may influence hormone responsiveness. In addition the impact on gene expression of having one strong and one weak affinity half-site, versus two moderate affinity half-sites, is not known. Even the prediction of the strength of a half-site based upon its sequence is not simple, since some base changes within the octamer are likely to be better tolerated than others. Nevertheless, near-perfect octamer half-sites have been identified in natural TREs (e.g. TGAGGTCA, the downstream half-site of the chick lysozyme IP6 TRE (40)) as have weak half-sites (e.g. GGAGGTGA, the upstream half-site of the rat α-myosin heavy chain DR4 TRE (41)). With the characterization of more natural TREs, it may become possible to identify a subset that shows sufficient variation in the half-site sequence as a marker for RXR dependence.
sequences, without variation in half-site orientation or number, and thus allow a direct comparison of the RXR dependence of gene expression.

In the current model of thyroid hormone-mediated gene activation, the major function of ligand is to induce conformational changes in nuclear receptor complexes that, in turn, regulate the interaction of associated factors carrying repression (corepressors) or activation (coactivators) activities (25). In the absence of ligand, corepressors associate with TR and repress transcription. Upon ligand binding, the corepressor dissociates and coactivators then bind the TR and activate transcription from the downstream promoter. The receptor-mediated ligand-independent gene expression from the single TREs used in this study is presumably due to the lack of nuclear receptor-specific corepressor proteins in yeast. In the absence of any corepressor, a constitutive activation function in TR is sufficient to activate reporter expression. In preliminary experiments we were unable to express functional nuclear receptor corepressor or silencing mediator for retinoid and thyroid hormone receptors in yeast and repress this constitutive activation. Since yeast also appear to lack nuclear receptor coactivators akin to SRC-1, liganded TR is functionally equivalent to unliganded TR and, therefore, unable to enhance promoter activity above the constitutive levels achieved in the absence of ligand. The small inhibitory effect of ligand seen in the absence of the coactivator SRC-1 likely reflects a conformational change in TR that alters the function of a constitutive TR activation domain. In addition, ligand binding may slightly lower the DNA binding affinity of the TR:TR homodimer and thereby decrease its ability to transactivate, although TR:RXR heterodimer binding to DNA is unaffected by the presence of ligand (37, 38).

In mammalian cells, unliganded TRs typically function as repressors of transcription from positive regulatory elements (23). It should be noted, however, that unliganded TRs also can activate transcription in mammalian cells, such as in the context of the thyrotropin releasing hormone and keratin promoters (42, 43). In fact, TR:TR homodimers may direct keratin gene expression, since TR:RXR heterodimers fail to bind the keratin TRE (43). Thus, the functioning of unliganded TRs as activators in S. cerevisiae has a precedent in mammalian cells and may not simply reflect an anomaly of mammalian nuclear receptor expression in yeast.

We were surprised to find that single response elements in our yeast reporter constructs responded qualitatively differently to ligand than reporter constructs bearing three response elements in tandem. We assumed that TRs which differed only in response element copy number would demonstrate proportional quantitative changes in reporter gene activation. This was not the case; ligand decreased the level of reporter gene expression from a single copy of 5DR4, whereas it stimulated reporter gene expression from the triple element (see Figs. 3A and 2). Ligand-dependent gene expression from the single element required coexpression of SRC-1 (discussed below), whereas ligand-dependent expression from the triple element did not. Thus, the ligand-dependent induction of gene expression from the triple response element may represent a qualitatively different mechanism by which nuclear receptors can activate gene expression than that engaged from the single TREs. Interactions among multiple TREs also may occur in certain mammalian genes, as the promoters for rat uncoupling protein (44) and human type I deiodinase (45) both contain multiple TREs. The association of nuclear hormone receptors on multiple response elements may permit protein interactions between receptor dimers that can be modulated by ligand-dependent conformational changes to promote gene activation. Our selection of single response elements for subsequent studies excluded the complications posed by multimerized response elements and allowed us to focus on the molecular aspects of transcriptional activation from single nuclear receptor dimer-DNA complexes.

The restoration of ligand-dependent gene activation from single response elements by SRC-1 (compare Figs. 3 and 5–7) demonstrates the importance of the coactivator protein in converting ligand binding by TRs into transcriptional activation of downstream promoters. Ligand-induced conformational changes in TRs bound to single response elements do not bring about gene activation through the receptor itself but rather through functional interactions with coactivators such as SRC-1. Importantly, SRC-1 did not alter the RXR requirements dictated by the composition of the TRE. Thus, SRC-1-mediated ligand-dependent gene activation from the suboptimal palindromic TRE (6PAL0) required the presence of RXR with TR, whereas SRC-1 activity from optimal TREs was maximal in the presence of TR alone.

Surprisingly, SRC-1 was able to support tric induction of gene expression only under certain circumstances. In general, SRC-1 was more potent in the presence of TR:RXR homodimers than TR:RXR heterodimers and also was more potent on (optimal) octamer half-sites than (suboptimal) hexamers. Thus, SRC-1 allowed for a large tric induction from 8DR4 in the presence of TR:RXR homodimers, a moderate induction from 8DR4 in the presence of TR:RXR heterodimers, a minimal induction from 6PAL0 in the presence of TR:RXR heterodimers, and no induction from 6DR4 or 6IP6. These data suggest that other coactivators may be required for thyroid hormone inductions involving heterodimers and/or suboptimal hexamer half-sites. Other coactivators that might be necessary for these T3 responses could include SRC-1 homologs (21, 46) or CREB binding protein (29). These data would imply that alterations in the availability of a coactivator could affect the T3 induction from only a subset of responsive genes within a cell.

How is it that the TRE sequence can influence the functional effects of SRC-1? Ikeda et al. (14) have demonstrated that the conformation of the TR:RXR heterodimer and its transcriptional activity can be modulated by the TRE to which it is bound. Therefore, the TR:RXR heterodimer may be functionally different in regard to its productive interaction with the coactivator SRC-1 when it binds different configurations and sequences of half-sites. Such TRE modulation of coactivator interaction and/or function suggests another level of regulation of TR-mediated gene expression.

This differential potency of SRC-1 on optimal versus suboptimal TREs is reminiscent of the promoter-specific coactivator function demonstrated in B-cells by the transcriptional coactivator Bob1 in conjunction with octamer transcription factors (47). Even though Oct-1:Bob1 complexes bind the octamer elements of the Ig-k and histone H2B promoters equally well, the H2B promoter exhibits reduced coactivation. Thus transcriptional coactivation appears to require more than just simple tethering of a coactivation domain to transcription factors. Other determinants of coactivator function, which may include protein conformation, appear to be encoded within the response element.

The nature of the attenuation of SRC-1 activity by RXR in the context of optimal TREs is unclear. SRC-1 was isolated in a two-hybrid screen using the progesterone receptor as the bait for interacting proteins (28). Since progesterone receptors interact with their palindromic response elements as homodimers, it is interesting to speculate that SRC-1 interacts with homodimeric receptor complexes differently than it does with TR:RXR heterodimers. This difference may be stoichio-
metric, or SRC-1 may have a higher affinity or more optimal conformation on homodimers, resulting in a greater degree of gene expression. In any case, the net effect may be to give the cell another means of regulating gene expression by thyroid hormone.

In conclusion we have demonstrated that TREs composed of suboptimal TR half-sites require RXR for TR-mediated ligand-independent and ligand-dependent gene expression. TREs composed of optimal TR half-sites are not dependent on RXR for TR-mediated ligand-independent or ligand-dependent gene expression. In addition, it is the presence of the coactivator SRC-1 that confers ligand dependence to TR-mediated gene activation from single response elements. SRC-1 activity varies with the composition of nuclear receptor dimers and the response elements to which the dimers are bound, showing the strongest effect with TR–TR homodimers bound to optimal direct repeat or palindromic TREs. Finally, both the composition of nuclear receptor dimers and the response elements to which the dimers are bound, showing the strongest effect with TR–TR homodimers bound to optimal direct repeat or palindromic TREs. Finally, both the composition and configuration of nuclear receptors that are competent to activate transcription in response to ligand are at least in part dictated by the response element architecture. Given the availability of RXR and SRC-1 in the mammalian nucleus, a continuum of response elements with variations in half-site sequence and orientation may provide the basis for a range of effects on different genes in response to the same thyroid hormone signal.

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David P. Olson and Ronald J. Koenig

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