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Thyroid Regulation of NADPH:Cytochrome P450 Oxidoreductase: Identification of a Thyroid-Responsive Element in the 5′-Flank of the Oxidoreductase Gene

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SUMMARY

The current study demonstrates that T_3-activated transcription of the NADPH:cytochrome P450 oxidoreductase (P450R) gene is dependent on the thyroid hormonal status of the animal, with both transcriptional and post-transcriptional pathways being important in regulating the cellular P450R mRNA level. The region required for transcriptional activation of the P450R gene by T_3 has been identified. Nuclear run-on experiments demonstrated that the effects of T_3 on P450R transcription are dependent on thyroid status, with a transcriptional enhancement obtained in T_3-treated hypothyroid rat liver (1.8-fold increase) but not in T_3-treated euthyroid animals. Transient cotransfection of P450R promoter/chloramphenicol acetyl transferase (CAT) constructs and the thyroid hormone receptor β1 (TRβ1) expression plasmid into rat hepatoma H4IIE cells resulted in a 2.4-fold induction of promoter activity that was both T_3 and TRβ1 dependent. Analysis of promoter deletion constructs identified a P450R-thyroid response region (P450R-TRE; bases, −564 to −536) containing three imperfect direct repeats of the thyroid response motif, AGGTCA. Mutational analysis further established that T_3 induction was dependent only on the upstream direct repeat, having the sequence AGGTGAgctgAGGCCA. Footprint analysis showed that all three motifs were protected by proteins present in rat liver nuclear extracts, and a direct interaction between P450R-TRE and T_3 receptors TRα1 and TRβ1 was demonstrated by gel-shift analysis. *In vitro* binding studies with P450R-TRE revealed the formation of heterodimeric complexes when TRα1 was coincubated with either the retinoic X receptor α or nuclear extract from rat liver, COS, or H4IIE cells. In addition, placement of the P450R-TRE upstream of the T_3-nonresponsive heterologous thymidine kinase promoter resulted in a 2.7-fold transcriptional enhancement that was both T_3 and TRβ1 dependent. Previous studies have demonstrated that T_3 augments P450R mRNA levels ~20–30-fold and ~12-fold, respectively, in hypothyroid and euthyroid rats. Hence, for the hypothyroid state, transcriptional and post-transcriptional events contribute to the T_3-induced mRNA increases; however, the marked increase in message level in T_3-treated euthyroid animals depends primarily on post-transcriptional pathways.

T_3 regulates numerous enzymes associated with hepatic metabolism, including the mixed function oxidase system (1). Composed of the cytochromes P450 and NADPH, P450R [NADPH:ferricytochrome oxidoreductase (EC 1.6.2.4)], this system is responsible for the initial metabolism of a wide range of lipophilic foreign compounds and functions directly in the biotransformation of many drugs as well as in the activation of chemical carcinogens. P450R serves as an internal electron transport chain, transferring electrons from NADPH to FAD to FMN and finally to cytochrome P450 and other enzymes such as heme oxygenase and cytochrome b_5 (2). Transfer of electrons from P450R to the cytochromes P450 is rate-limiting in many tissues, and alterations in the level of P450R have been shown to have an impact on the P450-dependent reactions (1).

In contrast to the cytochromes P450, which constitute a gene superfamily with ≥14 different mammalian families (3), P450R is encoded by a single gene localized in the mouse and human to chromosomes 6 and 7, respectively (4, 5). The rat gene spans >50 kb and has an untranslated first exon residing 30.5 kb upstream of the coding region (6, 7). Comparison of the amino acid sequence of P450R with other flavoproteins shows that the FMN-binding domain is homologous to the corresponding domains of the flavodoxins, whereas the FAD- and NADPH-binding domains are homol-

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ABBREVIATIONS: T_3, thyroid hormone; P450R, NADPH:cytochrome P450 reductase; TR, thyroid receptor; TRE, thyroid-responsive element; RXR, retinoid X receptor; TRAP, thyroid receptor auxiliary protein; CAT, chloramphenicol acetyl transferase; TK, thymidine kinase; rGH, rat growth hormone; rαMHC, rat α myosin heavy chain; rME, rat malic enzyme; DR, direct repeat; SDS, sodium dodecyl sulfate; bp, base pair(s).

ogous to ferridoxin-$NADP^+$ reductase (8). This high level of sequence conservation suggests that P450R arose through the fusion of two ancestral genes encoding these two flavoproteins.

Although P450R is required for the enzymatic activity of the cytochromes P450, it is independently regulated, with marked differences seen in both the extent and time course of induction by a variety of compounds (9, 10; for a review, see Ref. 2). To understand the modulation of cellular P450R levels, workers in one of our laboratories isolated the P450R promoter and have begun to determine factors affecting its transcriptional regulation. Unlike many other drug-metabolizing enzymes, including many of the cytochromes P-450, the P450R promoter does not contain either a TATA or a CCAAT box and is GC rich, possessing nine Sp1 consensus sites, of which only two are required to support basal transcription (11).

Studies on the hormonal regulation of rat liver cytochrome P-450 gene expression revealed that hypophysectomy decreased hepatic levels of P450R by ≤75% (1). Physiological replacement of T_3 but not other pituitary-dependent hormones in hypophysectomized animals substantially restored P450R levels, indicating that T_3 was required for full expression of P450R activity. Further studies demonstrated that T_3 administration to hypothyroid rats stimulated P450R mRNA production (~20–30-fold) while restoring protein and activity levels to normal (12). In contrast, T_3 treatment of erythroid animals produced a 12-fold increase in P450R mRNA without significantly affecting either protein or activity levels (12). Collectively, the data indicate that T_3 administration results in an increased message production regardless of thyroid status; however, the pathways regulating mRNA and protein levels remain to be defined.

The current study demonstrates that T_3 regulates cellular levels of P450R by both transcriptional and post-transcriptional mechanisms and identifies the thyroid response region of the promoter. An essential component of a T_3 transcriptional response is binding of the TR to specific recognition sequences in target genes, termed TREs, which consist of imperfect copies of a six nucleotide core binding site (5′-AGGTCA-3′) that can be organized into a variety of half-sites, including DRs, palindromes, and everted repeats (13, 14). The P450R-TRE identified in the current study is shown to contain imperfect DRs of the AGGTCA motif that bind to TRs with high affinity and sequence specificity. In addition, this region is able to confer T_3 responsiveness when transferred to an unresponsive heterologous promoter.

Materials and Methods

Animals. Adult male rats were maintained under standard conditions of light and temperature. The hormonal status of the animals was modulated as previously described (12). Hypothyroidism was achieved by administration of the antithyroid drug methimazole at a dose of 0.025% methimazole (w/v) in the drinking water for 16–24 days, and injection of hypothyroid (methimazole-treated) or euthyroid rats with a single intraperitoneal injection of T_3 (200 μg/100 g of body weight) resulted in rats that were hyperthyroid (12). Groups of rats were killed at the indicated times. Sprague-Dawley rats were used unless indicated otherwise.

Nuclear run-on transcription analysis. Isolation of liver nuclei from untreated and T_3-treated hypothyroid or euthyroid rats and run-on transcriptional analysis using α-^{32}P-UTP-labeled nascent RNA chains were carried out essentially as previously described (15, 16). Hybridization of labeled RNA transcripts to GeneScreen membranes (New England Nuclear Research Products, Boston, MA) containing linearized and heat-denatured P450R and S14 cDNAs was carried out at 65° for 48 hr in 4× SSPE (1× consists of 0.14 M NaCl, 0.88 mM NaH_2PO_4, 1 mM EDTA) containing 0.2% SDS, 1× Denhardt's solution, 10 μg/ml tRNA, and 1–3 × 10^6 cpm/ml ^{32}P-labeled RNA. Blots were then washed with 2× standard saline citrate (1× consists of 15 mM sodium citrate, pH 7.0, 150 mM NaCl) containing 0.1% SDS at 42° for 1 hr, with the buffer changed each 20 min. A second wash was conducted at 37° for 30 min with RNase solution (10 mM Tris·HCl, pH 7.5, containing 0.3 M NaCl, 40 mM EDTA, and 1 μg/ml RNase A). The final wash was at 42° for 1 hr in 0.1× standard saline citrate containing 0.1% SDS. The filters were air dried and subjected to analysis using a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Bands were quantified using ImageQuant software (Molecular Dynamics). S14 cDNA (3′-exon; pS14-C1) was kindly provided by Dr. D. Jump (Michigan State University).

Promoter constructs. The constructs p-488R, p-288R, and p-206R were prepared as previously described (11). Remaining constructs were ligated into pSVXBA1 and possessed an *Xba*I site at the 5′ end and a *Hin*dIII site at the 3′ end and contained 40 bp of the untranslated first exon and various regions of flanking sequence, with the number identifying the 5′ base. Plasmids p-584R and p-536R were generated through digestion of p-622R with *Pvu*II or *Taq*I, blunting where necessary, followed by ligation of *Xba*I linkers. Fragments were digested with *Xba*I/*Hin*dIII and ligated into pSVXBA1. Remaining plasmids were generated by polymerase chain reaction (17), with p-622R as a template, using the following primers (5′ to 3′ notation with the *Xba*I site given in lowercase letters): −575, CAGCTtctagaAGACATGAGATC; −564, AAGACAtctagaCACAGGTGAGC; and −552, CAGGTtctagaGAGGCCAGCAGC. At the 3′ end, all used a primer within the CAT gene: 5′-CGAGATTTTCAGGAGCTAAGGAAGC-3′. Fragments were digested with *Xba*I/*Hin*dIII, gel purified, and ligated into pSVXBA1. The heterologous promoter constructs were prepared by subcloning bases −564 to −532 of the P450R promoter or bases −185 to −164 of the rGH promoter upstream of the TK promoter in a Luciferase vector (18) to generate $pP450R_{TRE}$TKLuc and pGH_{TRE}TKLuc, respectively. Mutants p-584RM1, p-584RM2, and p-584RM3 were generated by sequential polymerase chain reaction (20). The top strand of the oligonucleotides used to generate the mutations were: p-584RM1, 5′-GATCAC<u>GAGCTC</u>GCTGAGGCCAGCAGCAGGTCG-3′; p-584RM2, 5′- GATCACAGGTGAGCTG<u>GAGCTC</u>GCAGCAGGTCG-3′; and p-584RM3, 5′- ACAGGTGAGCTGAGGCCAGCAG<u>CGAGCTC</u>AAACAC-3′(underlining indicates mutated bases). Mutants were placed upstream of a luciferase vector. All constructs were sequenced using Sequenase 2.0 (Amersham, Arlington Heights, IL).

Treatment of cells. COS cells and the rat hepatoma cell line H4IIE were grown in Dulbecco's modified Eagle's medium supplemented with 10% hormone-stripped fetal calf serum (19). Cells were transfected with 3 μg each of the promoter construct and an expression vector for TRβ1 (pCMD8-rTRβ1 (kindly provided by Dr. David Moore, Massachusetts General Hospital, Boston, MA) in the absence or presence of 10^{-7} M T_3 according to the DEAE dextran method as previously described (20). Transcriptional activity was measured by the CAT assay as optimized previously (20) or by the luciferase procedure (Promega, Madison, WI). Protein concentrations were determined according to the Lowry method. At least three separate transfection experiments were performed on triplicate dishes using two or more different DNA preparations. Ligand responsiveness of the various promoter constructs was expressed as the average of the T_3-treated to -untreated and presented as the average ± standard deviation of nine separate dishes.

Nuclear extract preparation and DNA/protein assays. Rat liver nuclear extract was prepared as previously described (11), and protein was determined according to the BCA method (Pierce, Rockford, IL). TRα1 (chicken) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

RXRα in pSG5 (21) was a generous gift of Janet Mertz (University of Wisconsin, Madison, WI). TRβ1 was excised from the pCMD8-TRβ1 vector and placed into pCITE 3b (Novagen, Madison, WI). TRβ1 and RXRα were then used for *in vitro* transcription/translation with the Single Tube Protein System 2 (Novagen). All reactions were run in duplicate with one translated in the presence of ^{35}S-methionine (Amersham) and the other translated with the unlabeled amino acid. SDS-polyacrylamide gel electrophoresis analysis demonstrated that >95% of the incorporated label was localized to a single protein band. For DNase protection assays, DNA fragments were isolated from the CAT constructs and labeled using Sequenase (Amersham). The coding strand of p-622R was labeled at the *Bam*HI site with α-^{32}P-dGTP after digestion with *Bam*HI/*Afl*III to generate a 367-bp fragment. The noncoding strand of p-622R was labeled at the *Hin*dIII site with α-^{32}P-dATP after digestion with *Hin*dIII/*Rsa*I to generate a 449-bp fragment. Reactions were carried out as previously described (11) and analyzed on a 6% sequencing gel. For gel-shift assays, reactions contained ~10^4 cpm DNA, 0.5–2 μg of poly(dI-dC), 5% glycerol, 10 mM Tris, pH 8.0, 60 mM KCl, 1 mM $MgCl_2$, and either 6–10 μg of rat liver nuclear extract or 2–4 μl of the appropriate rabbit reticulocyte lysate mixture. Reactions were incubated at room temperature for 20 min, and products were separated on a 5% nondenaturing polyacrylamide gel. For the competition studies, competitor probes were added 10 min before the addition of protein. The following oligonucleotides were used: ORTR, 5′-GATCACAGGTGAGCTGAGGCCAGCAGCAGGTCG-3′; DR4, 5′-CCCAGAATGTTAGGTCAAAGGAGGTCAAAATAG-3′; and ORU, 5′-CCAGGGCTGTACCAACAGCAGGGA-3′.

Results

T_3 regulation of the P450R gene: Transcriptional and post-transcriptional control. Treatment of euthyroid or hypothyroid rats with a receptor-saturating dose of T_3 stimulated a major induction of P450R mRNA, which peaked by 12 hr after hormone injection (12). To evaluate the contribution of T_3-induced gene transcription to the observed P450R mRNA increases, run-on transcription analyses were performed. T_3 induced a ~1.8-fold transcriptional activation of the P450R gene in hypothyroid rats by 8 hr (Fig. 1A). This transcriptional response was significantly lower than the ~20–30-fold increase in steady state liver P450R mRNA levels seen in these same animals (Ref. 12 and data not shown). It was also lower than the transcriptional induction of S14, a model T_3-regulated rat liver gene (22), obtained in the same nuclei (Fig. 1B). In contrast, little or no activation of P450R gene transcription was observed when T_3 was administered to euthyroid rats at the same receptor-saturating dose (Fig. 1A). This treatment is associated with a ~12-fold induction of liver P450R mRNA in the same livers (Ref. 12 and data not shown). A more detailed time course (1–24 hr) using nuclei from T_3-treated euthyroid animals in a separate series of experiments carried out with Sprague-Dawley rats confirmed the absence of detectable transcriptional activation in liver samples with induced levels of P450 mRNA (data not shown). These studies demonstrated that although T_3 can transcriptionally activate the P450R gene, the extent to which this activation contributes to the associated increases in P450R mRNA levels is dependent on the thyroid status of the animal. In the euthyroid state, post-transcriptional events are primarily responsible for the mRNA increase, whereas in the hypothyroid animals, both transcriptional and post-transcriptional pathways are operative.

Identification of the T_3 responsive region. To identify *cis*-acting sequences responsible for conferring T_3 transcriptional regulation, a series of 5′ deletion constructs of the

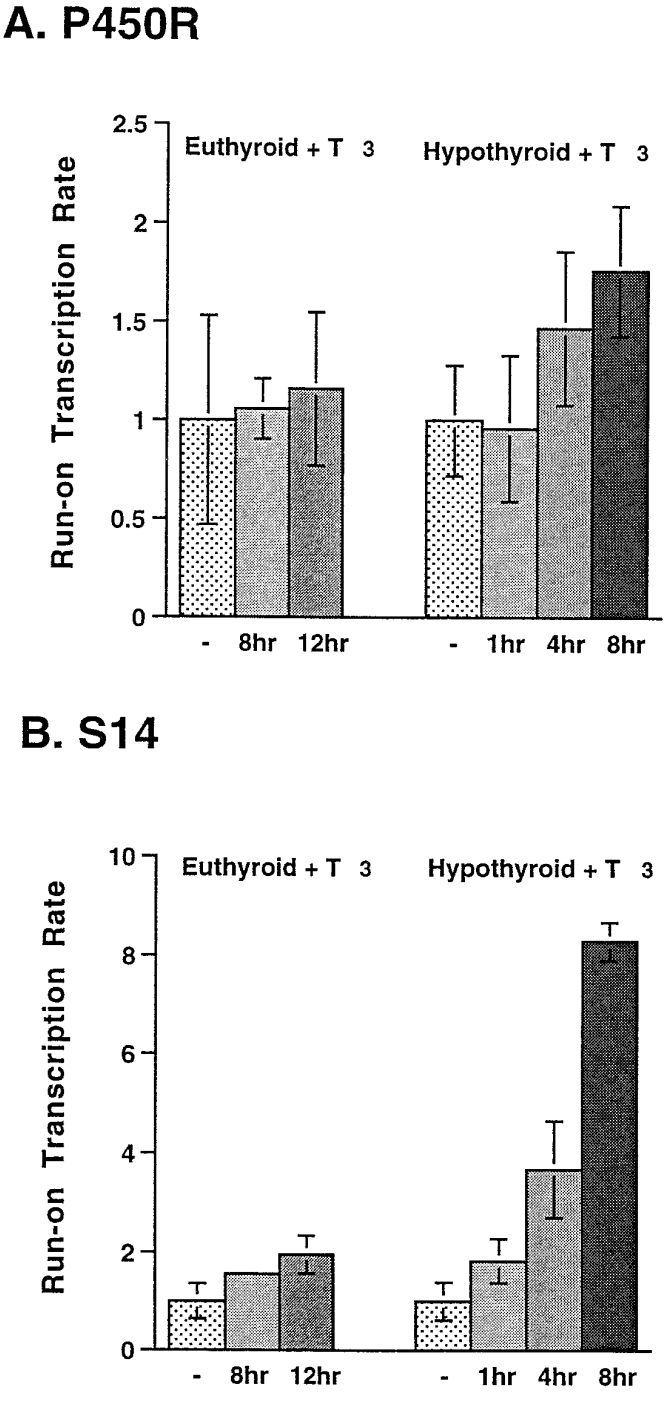

Fig. 1. Nuclear run-on transcriptional analysis of P450R (A) and S14 (B) genes in rat liver. Nuclei isolated from untreated and T_3-heated hypothyroid and euthyroid rat liver were incubated in the presence of [α^{32}P]UTP to elongate nascent RNA transcripts. Labeled RNA was then isolated and hybridized to filters containing P450R or S14 probes as described in Materials and Methods. Shown are run-on transcription rates relative to hormone-free controls determined by PhosphorImager analysis. Results are expressed as mean ± standard deviation for three individual rats/treatment group.

P450R promoter linked to the CAT gene were analyzed in transient transfection studies. When the rat hepatoma cell line H4IIE was transfected with p-622R in the presence or absence of T_3, no induction was noted (data not shown). However, cotransfection with an expression vector encoding

rat TRβ1 resulted in a ~2.4-fold increase in promoter activity that was both hormone and receptor dependent (Fig. 2). No induction was observed in the absence of hormone or when a β-galactosidase expression plasmid was cotransfected in place of TRβ1. The 2.4-fold induction observed in cultured cells corresponds to the effects of T_3 on P450R transcription in hypothyroid animals (Fig. 1A). Transient transfection of longer constructs, containing $\leq$1.5 kb of 5′ flanking region, revealed a similar response to hormone (data not shown), indicating that the proximal 622 bp is sufficient for induction by T_3. In contrast, constructs p-488R, p-405R, and p-206R were unresponsive to T_3, indicating that the hormone-responsive element half-site (TGACCT) previously identified between bases −213 and −218 (7) was not involved in T_3 regulation (Fig. 2) and further narrowing the T_3 responsive region to the 134 bp between −622 and −488. Although T_3 produced equivalent inductive responses with p-622R and p-584R, constructs p-575R and p-564R showed a similar, although slightly lower, induction (~1.8-fold). Interestingly, deletion of bases −564 to −552 (p-552R) resulted in a total loss of T_3 responsiveness, localizing the hormone responsive region to the promoter segment defined by bases −564 to −552.

Core TRE sequences usually consist of two or more copies of the hexameric sequence AGGTCA arranged in various orientations (13, 14). Sequence analysis of bases −561 to −535 of the P450R T_3 responsive region revealed three imperfect DRs having the general TRE motif AGGTCA and the specific sequences and spacing of AGGTgA $(N)_4$ AGGcCA $(N)_5$ AGGTCg [deviations from the AGGTCA consensus shown in lower case] (Fig. 3). The presence of these motifs related to the functional importance of this region; T_3 induction was maintained in p-564R, which contained all three motifs, whereas p-552R, in which the motif nearest the 5′ end was deleted, lost responsiveness to T_3 (Fig. 2). This is a key observation because it identifies the AGGTGA sequence as being required for the full T_3 response. The region containing the three motifs was designated the P450R-TRE. Comparison of other naturally occurring TREs to the P450R-TRE illustrates the high degree of variation noted among different responsive elements (Fig. 3).

Multiple DNA/protein interactions at the P450R-TRE. Protein binding sites in the T_3 responsive region of the P450R promoter were identified by DNase footprint analysis (Fig. 4). A probe that encompassed bases −622 to −405 of the P450R promoter was labeled on the coding strand and incubated with liver nuclear extract from euthyroid rats (Fig. 4, *lane 2*). Several protected regions were observed, including bases −568 to −545, which contained the two upstream motifs AGGTgA (N4) AGGcCA, and bases −542 to −528, which covered the third motif AGGTCg. DNase footprint analysis of the noncoding strand also demonstrated protein binding over these motifs as bases −575 to −552 and −548 to −533 were protected from digestion, as well as an additional binding site from −530 to −507 (Fig. 4, *lanes 5 and 6*). Examination of liver nuclear extract from rats rendered hypothyroid by treatment with methimazole produced similar results (Fig. 4, *lane 7*). This finding supports previous work, which established that TR is localized to the nucleus and can be bound to DNA in the absence of ligand (23–25). Collectively, the cell culture and footprint studies establish that the P450R-TRE is functionally important and that nuclear proteins bind to all three motifs.

To directly assess whether TR can bind to the P450R-TRE, a double-stranded oligonucleotide that encompassed all three motifs (bases −564 to −532) was synthesized and used as a probe in gel-shift assays. TRs can bind to TREs as monomers, homodimers. or heterodimers, depending on the sequence and orientation of the motifs and whether additional proteins are present (13). Previous studies have demonstrated that the monomeric form displays a faster mobility in gel-shift analyses compared with the dimeric form of the receptor (24, 26). Incubation of labeled P450R-TRE with TRα1 resulted in the formation of two retarded complexes (Fig. 5A, *lanes 3–5*). At low concentrations of TRα1, only the faster migrating monomeric complex was observed (Fig. 5A, *lane 2*), and as additional TRα1 was added, the slower mobility homodimeric complex was generated (Fig. 5A, *lanes 3–5*). Incubation of labeled P450R-TRE with both TRα1 and rat liver nuclear extract resulted in the loss of the monomeric complex, whereas at the same time a broad slower mobility band was produced that overlapped the homodimer band (Fig. 5A, *lane*

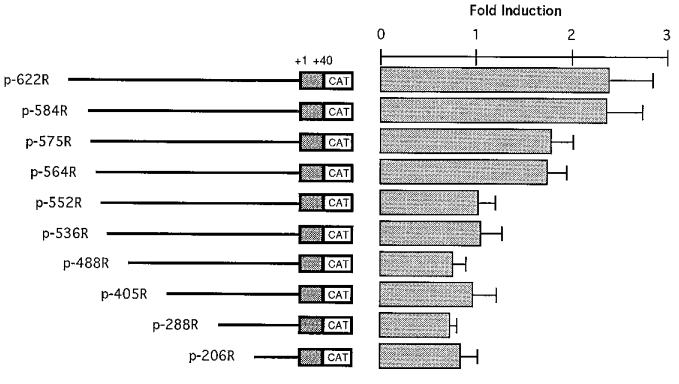

Fig. 2. Transcriptional induction by T_3 of P450R-CAT constructs. P450R-CAT promoter deletion constructs were cotransfected with the T_3 receptor expression vector pCDM8-rTRβ into H4IIE cells. Results are presented as fold induction in the presence versus the absence of 10^{-7} M T_3. *Bars*, average ± standard deviation of at least nine separate dishes.

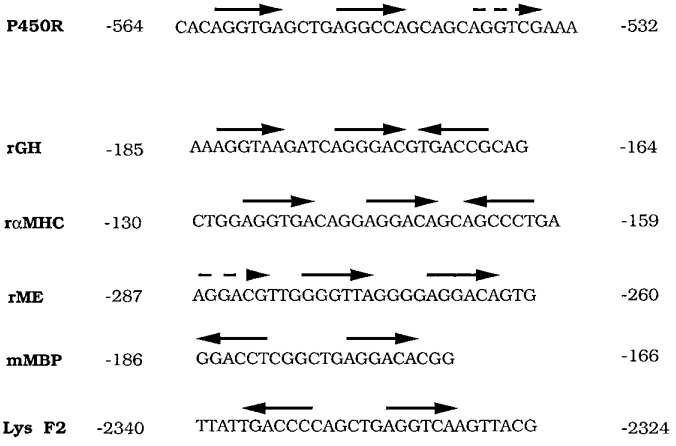

Fig. 3. Comparison of the nucleotide sequence of various T_3 receptor binding sites with the P450R-TRE. The sequence between −564 and −532 of the P450R promoter is shown. The sequences of various native TREs are from the promoters of the following genes: rGH, rαMHC, rME (Ref. 37 and references therein), mouse myelin basic protein (*mMBP*) (43), and chicken lysozyme F2 (*Lys F2*) (44). *Arrows*, positions of the AGGTCA motifs indicating the orientation of the repeat. *Dashed arrows*, nonfunctional motifs.

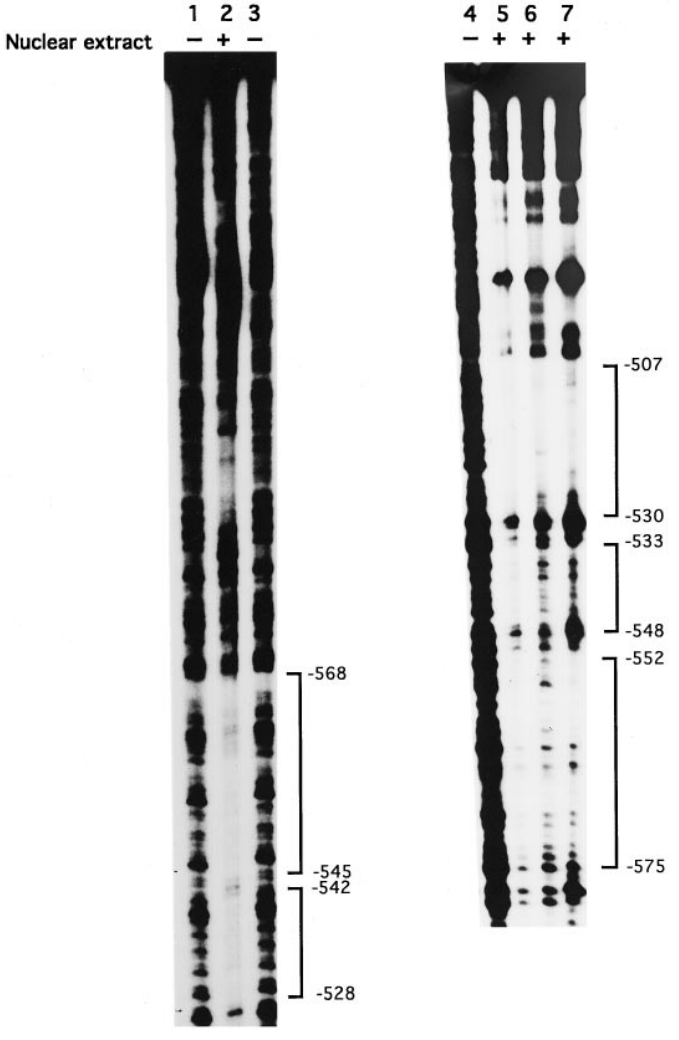

Fig. 4. DNase I protection analysis of the T_3 responsive region. For the coding strand (*lanes 1–3*), a 367-bp *Bam*HI/*Afl*III fragment was labeled at the *Bam*HI site with α-^{32}P-dGTP, whereas for the noncoding strand, a 449-bp *Hin*dIII/*Rsa*I fragment was labeled at the *Hin*dIII site with α-^{32}P-dATP (*lanes 4–7*). Probes were incubated in the presence or absence of rat liver nuclear extract before digestion with DNase I. *Lanes 1, 3, and 4*, no protein. *Other lanes* contained 50 μg of rat liver nuclear extract isolated from 6-week-old euthyroid rats (*lanes 2 and 5*), 10-week-old euthyroid rats (*lane 6*), or rats treated for 24 days with the antithyroid drug methimazole (*lane 7*). *Numbers*, regions of protection indicating the position relative to the transcription start site.

6). Rat liver nuclear extract alone also exhibited similar complex formation, although the bands were not as intense (Fig. 5A, *lane 7*). Rat liver nuclear extract contains TRs and a number of TRAPs that form TR/TRAP heterodimers (13, 21, 27–29). Thus, it seems that the P450R-TRE can directly bind TRα1 as both a monomer and a homodimer, and heterodimeric complexes will form in the presence of rat liver nuclear extract.

The functionally active form of TR is thought to be the TR/TRAP heterodimer (21, 27–30). Transfection experiments were carried out in both H4IIE and COS cells, each of which appeared to be deficient in TR, because cotransfection of receptor was required to elicit a hormonal response. The ability of extracts from these cells to form a gel-shift complex with P450-TRE was evaluated (Fig. 5B). Each extract produced a distinct pattern of complex formation, which is consistent with the observation that certain cells differentially express TRAPs, which allows the formation of specific TR/TRAP heterodimers (31). In comparison to TR homodimers, TR/TRAP heterodimers show an increased binding affinity to TREs, with the major TR in rat liver being TRβ1 (13) and the most abundant TRAP being RXRα (29). Incubation of labeled P450R-TRE with TRα1 and RXRα resulted in the formation of a new complex (Fig. 6, *lane 3*), which was not present when the probe was incubated alone with either TRα1, RXRα, or unprogrammed lysate (Fig. 6, *lanes 2, 6, and 7*, respectively),

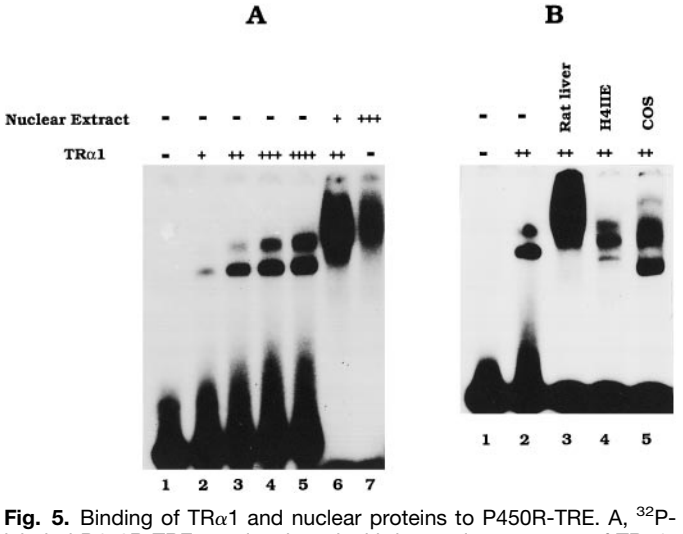

Fig. 5. Binding of TRα1 and nuclear proteins to P450R-TRE. A, ^{32}P-labeled P450R-TRE was incubated with increasing amounts of TRα1-GST fusion protein (0.2–0.8 μg; *lanes 2–5*, respectively). Probe was incubated with 0.4 μg of TRα1 and 6 μg of rat liver nuclear extract (*lane 6*) or 18 μg of rat liver nuclear extract alone (*lane 7*). B, The P450R-TRE probe was incubated with 0.4 μg of TRα1 (*lanes 2–5*) and 6 μg of nuclear extract from rat liver (*lane 3*), H4IIE cells (*lane 4*), or COS cells (*lane 5*).

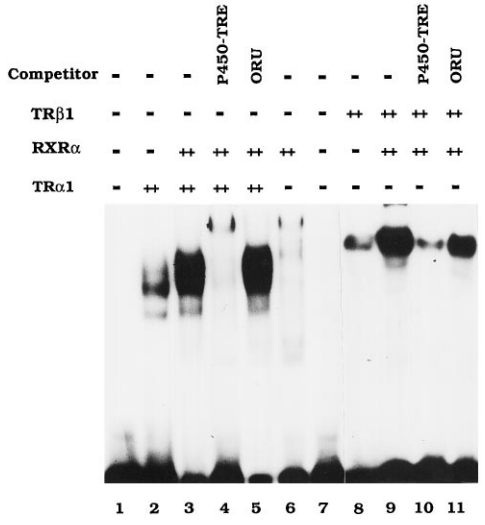

Fig. 6. P450R-TRE/receptor interactions. Binding of TRα1, TRβ1, and RXRα to P450R-TRE. RXRα and TRβ1 were synthesized by an *in vitro* transcription/translation system. The ^{32}P-labeled P450R-TRE oligonucleotide probe was incubated with 2 μl of either TRα1 (*lane 2*) or TRβ1 (*lane 8*) or in combination with 2 μl of RXRα (*lanes 3–5 and 9–11*). A 50-fold excess of cold competitor, either self (P450R-TRE; lanes 4 and 10), or non-specific oligonucleotide (ORU; *lanes 5 and 11*) was added to the reactions 10 min before the addition of probe. *Lane 6*, 2 μl of RXRα. *Lane 7*, 2 μl of unprogrammed rabbit reticulocyte lysate. Reactions were analyzed on a 4% nondenaturing polyacrylamide gel.

indicating that the TRα/RXRα heterodimer bound to the P450R-TRE. Competition studies demonstrated the specificity of these interactions, as a 50-fold excess of self-inhibited formation of the labeled complex (Fig. 6, *lane 4*), whereas a 50-fold excess of a nonspecific competitor (ORU) did not diminish complex formation (Fig. 6, *lane 5*). Similar *in vitro* binding experiments demonstrated that TRβ1 also bound to P450R-TRE (Fig. 6, *lane 8*). The addition of RXRα produced a higher affinity complex whose formation was inhibited by a 50-fold excess of either self but not by a nonspecific competitor (Fig. 6, *lanes 9–11*), also suggesting TRβ/RXRα could bind as a heterodimer to P450R-TRE.

P450R-TRE confers hormone responsiveness to a heterologous promoter. To evaluate whether P450-TRE could activate a nonresponsive heterologous promoter, three copies of P450R-TRE were placed upstream of the TK promoter in a luciferase reporter plasmid (p$P450R_{TRE}$TKLuc). As a positive control, two copies of the rGH TRE (Fig. 3) were also inserted upstream of the same promoter. Because the basal activity of the heterologous promoter was low in H4IIE cells, constructs were transfected into COS cells. COS cells retain a number of TRAPs, including RXR, and have been used previously to characterize TREs (31). T_3 treatment of cells transfected with p$P450R_{TRE}$TKLuc resulted in a 2.7-fold increase in reporter gene activity, whereas T_3 stimulation of pGH_{TRE}TKLuc increased promoter activity 3.5-fold (Table 1). Both responses were dependent on cotransfection of TRβ1 (data not shown). These results demonstrate that the P450R-TRE is able to augment transcription of a heterologous promoter in a receptor- and hormone-dependent manner.

Mutational analysis demonstrates the 5′ DR is essential for the T_3 response. Although considerable variation has been noted among naturally occurring TREs (Fig. 3), the length of the spacer between DRs plays an important role in achieving a selective hormonal response, with spacings of 3, 4, and 5 bp conferring preferential transcriptional responses to receptors for vitamin D, T_3, and retinoic acid, respectively (32, 33). To more fully define the sequences required for induction, each motif was separately modified to abolish similarity to an AGGTCA consensus. Mutations were generated in p-584R, a construct that displayed maximum response to T_3. Modification of the 5′ (p-584RM1) and middle (p-584RM2) motifs resulted in the loss of induction by T_3 (Fig. 7). In contrast, p-584RM3, in which the 3′ motif was mutated, retained responsiveness to the hormone. Mutations did not decrease basal activity (data not shown). These results correlate with those obtained for the deletion construct p-552R, which showed a loss of T_3 induction on the removal of the 5′ motif of the TRE. Thus, the TRE of the P450R gene is composed of an imperfect DR4 with the sequence AGGTGAgctgAGGCCA.

TABLE 1
Regulation of the TK promoter by P450R-TRE

Construct	T_3/control[a]
TKLUC	1.2 ± 0.2
GH_{TRE}-TKLUC	3.5 ± 0.8
$P450R_{TRE}$TKLUC	2.7 ± 0.5

[a] Constructs were transiently transfected into COS cells in the presence or absence of T_3. Values are the average fold induction ± standard deviation of nine separate dishes.

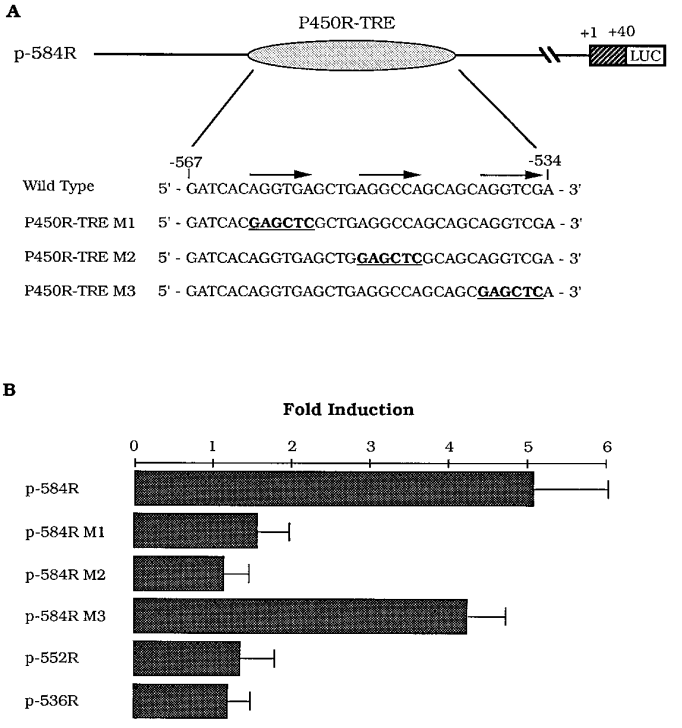

Fig. 7. Mutational analysis of the DRs of the P450R-TRE. A, DRs of the P450R-TRE were individually mutated in the p-584R construct. *Bold and underlined*, mutated sequences. B, p-584R wild-type and mutant promoter constructs were cotransfected with the T_3 receptor expression vector pCDM8-rTRβ into H4IIE cells. Results are presented as fold induction (T_3-treated/control). *Bars*, average ± standard deviation of at least nine separate dishes.

Discussion

Previously, it was demonstrated that T_3 could greatly stimulate the expression of P450R mRNA (12). The current study demonstrates that both hormone-dependent transcriptional and post-transcriptional events contribute to this response. Studies on the transcriptional regulatory mechanism identified an element, designated P450R-TRE, that is crucial for T_3-stimulated, TR-dependent P450R gene transcription. This element is located between bases −564 to −535 and contains three imperfect DRs of the AGGTCA motif, two of which (−561 to −546) were shown to be essential for the T_3 response. DNase footprinting and gel mobility shift studies demonstrated that not only do proteins present in rat liver nuclear extract bind to this regulatory element but also a direct interaction is observed between P450R-TRE and both TRα1 and TRβ1. In addition, placement of P450R-TRE upstream of a heterologous promoter confers T_3 sensitivity to a nonresponsive promoter.

The direct effects of T_3 on target genes are mediated by the TRs that bind specific responsive elements. These receptors are members of the steroid/thyroid superfamily of nuclear transcription factors that bind to copies of the core recognition motif AGGTCA found in several orientations, including DR, palindrome, and everted repeat (14). Multiple isoforms of TR exist; however, although the receptors β1, β2, and α1 display characteristic patterns of developmental, tissue-specific, and hormonal regulation, all have been found to comparably bind both DNA and T_3, form heterodimers, and *trans*-activate responsive genes (13). Indeed, in the current

study, both TRα1 and TRβ1 bind to the P450R-TRE, and TRβ1, which is the major TR found in rat liver (13), can *trans*-activate the P450R promoter constructs.

The P450-TRE element not only was able to directly bind to TRα1, TRβ1, and rat liver nuclear extract but also formed heterodimeric complexes on coincubation of TRα1 and either RXRα or nuclear extract from rat liver, H4IIE, or COS cells (Figs. 5 and 6). Many studies have demonstrated that TRs can bind to TREs as monomers, homodimers, and heterodimers by association with TRAPs, but the specific complex that binds TRE is influenced by factors such as sequence and arrangement of hexamer motifs, availability and relative content of accessory proteins, presence of ligand, and receptor phosphorylation status (for a review, see Ref. 13). Using a variety of TREs, TR seems to bind preferentially to DNA as a heterodimer with RXR (21, 27–30) and other nuclear proteins (34–36), and several studies have indicated that the functionally active form of TR is the TR/RXR heterodimer (21, 27–30). However, functional augmentation of the T_3 response by RXR seems to be both cell and element specific (31). Not only is RXRα the major TRAP in rat liver extract (29), but it is also found in COS nuclear extract (31).

The P450R T_3 responsive region displays a complex motif arrangement containing three imperfect DRs separated by 4 and 5 bp, respectively (Fig. 3). Deletion analysis of the P450R promoter region indicates that the 5′-most motif is critical for T_3 responsiveness (Fig. 2). However, rat liver nuclear extract protected all three motifs in DNase footprint analysis (Fig. 4), and preliminary results have also indicated that if the region is subdivided into P450R-DR4 (bases −567 to −541) or P450R-DR5 (bases −555 to −528), TRα1 will bind to either DR in gel-shift experiments (data not shown). Although the length of spacer between DRs seems to play an important role in achieving a selective hormonal response, with a spacing of four conferring preferential transcriptional response to TR (32, 33), considerable variation is noted among naturally occurring TREs (Fig. 3). For example, mutational analysis of the rGH, rαMHC, and rME TREs demonstrate that although all three motifs of the rGH and rαMHC genes must be intact for maximal T_3 induction, only the two motifs (Fig. 3, *solid arrows*) are essential for hormone responsiveness of the rME TRE (37). Mutational analysis of the P450R T_3 responsive region indicated that like the rME TRE, only the DR4 element is essential for the T_3 response (Fig. 7).

Recently, it was reported that for an idealized DR, the optimum TRE actually consists of an 8-bp sequence (TAAGGTCA), not a hexamer, and equivalently strong T_3 responses were conferred on octamer DRs with spacing of 3, 4, or 5 bp (38), indicating that TRE spacing may not be the only factor to dictate hormone sensitivity. Although P450R-TRE does not contain this particular octameric sequence, the precise sequence requirements within this 8-bp motif have not been strictly defined. In addition, the P450R promoter region footprint observed with rat liver nuclear extract extends upstream of the actual TRE motif; the motif begins at −561, whereas the footprint extends 5′ to base −575. Whether this is due to an extended TR-binding site or binding of an additional protein(s) is not clear; however, the fact that protein binding occurs further upstream of the P450R-TRE may explain the small but significant drop in T_3 induction found in constructs p-575R and p-564R.

Results from this study clearly demonstrate that T_3-activated transcription of the P450R gene depends on the T_3 status of the animal. The 1.8-fold increase in transcriptional activity seen in T_3-treated hypothyroid rats is not directly proportional to the >20-fold increase in P450R mRNA seen in the same animals (Ref. 12 and data not shown), indicating that both transcriptional and post-transcriptional events contribute to the observed mRNA increase. Similarly, the 12-fold increase in P450R mRNA in T_3-treated euthyroid rats is not accompanied by an increased transcriptional response, suggesting that post-transcriptional pathways regulated by T_3 account for the increased message level. Together, these findings suggest that the P450R mRNA increases associated with the transition from the hypothyroid state to the euthyroid state are largely transcriptional and likely mediated by P450R-TRE, whereas the increases associated with hyperthyroidism are mainly post-transcriptional. Other T_3-regulated genes also show a dependence on hormonal state for transcriptional activation. For example, significant transcriptional activation of the S14 gene was observed only in T_3-treated hypothyroid rats (16), a finding that was confirmed in the current study (Fig. 1). Multiple levels of control, including regulation of protein half-life, mRNA stability and rates of transcriptional initiation, have been reported for other T_3 responsive genes (for a review, see Ref. 34). Although some genes display a single level of control, such as the RXRβ and RXRγ genes (39), T_3 can affect many genes at multiple levels. For example, although the S14 gene is transcriptionally regulated by T_3, its mRNA is also rapidly stabilized (40). Malic enzyme also shows both a T_3-dependent transcriptional activation and a decrease in the rate of mRNA degradation (41). In addition, T_3 transcriptionally activates the rat cardiac myosin heavy chain gene and affects two different post-transcriptional events, including alternative splicing and variable 3′ end formation (42). These or other post-transcriptional mechanisms could be responsible for the ~12-fold increase in P450R mRNA observed on T_3 treatment of hypothyroid rats.

In addition to disease state, physiological and environmental factors, such as pregnancy, malnutrition, environmental temperature, and stress, can affect the levels of circulating T_3. P450R is thought to be rate limiting for many liver P450-catalyzed reactions, and alterations in the P450R levels in response to changes in T_3 levels are thus likely to affect P450-dependent metabolism of numerous drugs, carcinogens, and other substrates (1). Further studies of the underlying molecular basis for regulation of P450R by T_3 will allow a better prediction of the effects of hormonal status on the metabolism of xenobiotics as well as endogenous steroids and other P450 substrates.

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References

1. Waxman, D. J., J. J. Morrissey, and G. A. Leblanc. Hypophysectomy differentially alters P-450 protein levels and enzyme activities in rat liver: pituitary control of hepatic NADPH cytochrome P-450 reductase. *Mol. Pharmacol.* **35:**519–525 (1989).
2. Shen, A. L., and C. B. Kasper. Protein and gene and regulation of NADPH cytochrome P450 oxidoreductase. *Handb. Exp. Pharmacol.* **105:**35–59 (1993).
3. Nelson, D. R., L. Koymans, T. Kamataki, J. J. Stegeman, R. Feyereisen, D.

J. Waxman, M. R. Waterman, O. Gotoh, M. J. Coon, R. W. Estabrook, I. C. Gunsalus, and D. W. Nebert. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* **6:**1–42 (1996).
4. Simmons, D. L., P. A. Lalley, and C. B. Kasper. Chromosomal assignments of genes coding for components of the mixed-function oxidase system in mice: genetic localization of the cytochrome P-450PCN and P-450PB gene families and the NADPH-cytochrome P-450 oxidoreductase and epoxide hydratase genes. *J. Biol. Chem.* **260:**515–521 (1985).
5. Yamano, S., T. Aoyama, O. W. McBride, J. P. Hardwick, H. V. Gelboin, and F. J. Gonzalez. Human NADPH-P450 oxidoreductase: complementary DNA cloning, sequence and vaccinia virus-mediated expression and localization of the CYPOR gene to chromosome 7. *Mol. Pharmacol.* **36:**83–88 (1989).
6. Porter, T. D., T. W. Beck, and C. B. Kasper. NADPH-cytochrome P-450 oxidoreductase gene organization correlates with structural domains of the protein. *Biochemistry* **29:**9814–9818 (1990).
7. O'Leary, K. A., T. W. Beck, and C. B. Kasper. NADPH cytochrome P-450 oxidoreductase gene: identification and characterization of the promoter region. *Arch. Biochem. Biophys.* **310:**452–459 (1994).
8. Porter, T. D., and C. B. Kasper. NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins. *Biochemistry* **25:**682–687 (1986).
9. Hardwick, J. P., F. J. Gonzalez, and C. B. Kasper. Transcriptional regulation of rat liver epoxide hydratase, NADPH-cytochrome P-450 oxidoreductase, and cytochrome P-450b genes by phenobarbital. *J. Biol. Chem.* **258:**8081–8085 (1983).
10. Simmons, D. L., P. McQuiddy, and C. B. Kasper. Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoids: transcriptional and post-transcriptional regulation. *J. Biol. Chem.* **262:**326–332 (1987).
11. O'Leary, K. A., P. McQuiddy, and C. B. Kasper. Transcriptional regulation of the TATA-less NADPH cytochrome P-450 oxidoreductase gene. *Arch. Biochem. Biophys* **330:**271–280 (1996).
12. Ram, P. A., and D. J. Waxman. Thyroid hormone stimulation of NADPH P450 reductase expression in liver and extrahepatic tissues: regulation by multiple mechanisms. *J. Biol. Chem.* **267:**3294–3301 (1992).
13. Lazar, M. A. Thyroid hormone receptors: multiple forms, multiple possibilities. *Endocr. Rev.* **14:**184–193 (1993).
14. Glass, C. K. Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocr. Rev.* **15:**391–407 (1994).
15. Tukey, R. H., and S. T. Okino. Quantitation of related gene products by nuclear run-on and Northern blot analysis. *Methods Enzymol.* **206:**284–290 (1991).
16. Jump, D. B. Rapid induction of rat liver S14 gene transcription by thyroid hormone. *J. Biol. Chem.* **264:**4698–4703 (1989).
17. Cormack, B. Directed mutagenesis using the polymerase chain reaction, in *Current Protocols in Molecular Biology* (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Strohl, eds.). Wiley Interscience, New York, 8.5.1–8.5.9 (1994).
18. Huss, J. M., S. I. Wang, A. Astrom, P. McQuiddy, and C. B. Kasper. Dexamethasone responsiveness of a major glucocorticoid-inducible CYP3A gene is mediated by elements unrelated to a glucocorticoid receptor binding motif. *Proc. Natl. Acad. Sci. USA* **93:**4666–4670 (1996).
19. Sammuels, H. H., F. Stanley, and J. Casanova. Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* **105:**80–85 (1979).
20. Bell, P. A., C. N. Falany, P. McQuiddy, and C. B. Kasper. Glucocorticoid repression and basal regulation of the epoxide hydrolase promoter. *Arch. Biochem. Biophys.* **279:**363–369 (1990).
21. Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J. Y. Chen, A. Staub, J. M. Garnier, S. Mader, and P. Chambon. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. *Cell* **68:**377–395 (1992).
22. Jump, D. B., A. Veit, V. Santiago, G. Lepar, and L. Herberholz. Transcriptional activation of the rat liver S14 gene during postnatal development. *J. Biol. Chem.* **263:**7254–7260 (1988).
23. Lazar, M. A., T. J. Berrodin, and H. P. Harding. Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor. *Mol. Cell. Biol.* **11:**5005–5015 (1991).
24. Forman, B. M., J. Casanova, B. M. Raaka, J. Ghysdael, and H. H. Samuels. Half-site spacing and orientation determines whether thyroid hormone and retinoic acid receptors and related factors bind to DNA response elements as monomers, homodimers, or heterodimers. *Mol. Endocrinol.* **6:**429–442 (1992).
25. Dalman, F. C., R. J. Koenig, G. H. Perdew, E. Massa, and W. B. Pratt. In contrast to the glucocorticoid receptor, the thyroid hormone receptor is translated in the DNA binding state and is not associated with hsp90. *J. Biol. Chem.* **265:**3615–3618 (1990).
26. Williams, G. R., J. W. Harney, B. M. Forman, H. H. Samuels, and G. A. Brent. Oligomeric binding of T3 receptor is required for maximal T3 response. *J. Biol. Chem.* **266:**19636–19644 (1991).
27. Bugge, T. H., J. Pohl, O. Lonnoy, and H. G. Stunnenberg. RXR *alpha*, a promiscuous partner of retinoic acid and thyroid hormone receptors. *EMBO J.* **11:**1409–1418 (1992).
28. Zhang, X. K., B. Hoffmann, P. B. Tran, G. Graupner, and M. Pfahl. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. *Nature (Lond.)* **355:**441–446 (1992).
29. Sugawara, A., P. M. Yen, D. S. Darling, and W. W. Chin. Characterization and tissue expression of multiple triiodothyronine receptor-auxiliary proteins and their relationship to the retinoid X-receptors. *Endocrinology* **133:**965–971 (1993).
30. Kliewer, S. A., K. Umesono, D. J. Mangelsdorf, and R. M. Evans. Retinoid X receptor interacts with nuclear receptors in retinoic acid, thyroid hormone and vitamin D3 signalling. *Nature (Lond.)* **355:**446–449 (1992).
31. Hsu, J. H., A. M. Zavacki, J. W. Harney, and G. A. Brent. Retinoid-X receptor (RXR) differentially augments thyroid hormone response in cell lines as a function of the response element and endogenous RXR content. *Endocrinology* **136:**421–430 (1995).
32. Naar, A. M., J. M. Boutin, S. M. Lipkin, V. C. Yu, J. M. Holloway, C. K. Glass, and M. G. Rosenfeld. The orientation and spacing of core DNA-binding motifs dictate selective transcriptional responses to three nuclear receptors. *Cell* **65:**1267–1279 (1991).
33. Umesono, K., K. K. Murakami, C. C. Thompson, and R. M. Evans. Direct repeats as selective response elements for the thyroid hormone, retinoic acid, and vitamin D3 receptors. *Cell* **65:**1255–1266 (1991).
34. Glass, C. K., and J. M. Holloway. Regulation of gene expression by the thyroid hormone receptor. *Biochim. Biophys. Acta* **1032:**157–176 (1990).
35. Burnside, J., D. S. Darling, and W. W. Chin. A nuclear factor that enhances binding of thyroid hormone receptors to thyroid hormone response elements. *J. Biol. Chem.* **265:**2500–2504 (1990).
36. Murray, M. B., and H. C. Towle. Identification of nuclear factors that enhance binding of the thyroid hormone receptor to a thyroid hormone response element. *Mol. Endocrinol.* **3:**1434–1442 (1989).
37. Brent, G. A., G. R. Williams, J. W. Harney, B. M. Forman, H. H. Samuels, D. D. Moore, and P. R. Larsen. Capacity for cooperative binding of thyroid hormone (T3) receptor dimers defines wild type T3 response elements. *Mol. Endocrinol.* **6:**502–514 (1992).
38. Katz, R. W., J. S. Subauste, and R. J. Koenig. The interplay of half-site sequence and spacing on the activity of direct repeat thyroid hormone response elements. *J. Biol. Chem.* **270:**5238–5242 (1995).
39. Mano, H., R. Mori, T. Ozawa, K. Takeyama, Y. Yoshizawa, R. Kojima, Y. Arao, S. Masushige, and S. Kato. Positive and negative regulation of retinoid X receptor gene expression by thyroid hormone in the rat: transcriptional and post-transcriptional controls by thyroid hormone. *J. Biol. Chem.* **269:**1591–1594 (1994).
40. Narayan, P., and H. C. Towle. Stabilization of a specific nuclear mRNA precursor by thyroid hormone. *Mol. Cell. Biol.* **5:**2642–2646 (1985).
41. Song, M. K., B. Dozin, D. Grieco, J. E. Rall, and V. M. Nikodem. Transcriptional activation and stabilization of malic enzyme mRNA precursor by thyroid hormone. *J. Biol. Chem.* **263:**17970–17974 (1988).
42. Sindhwani, R., F. Ismail-Beigi, and L. A. Leinwand. Post-transcriptional regulation of rat α cardiac myosin heavy chain gene expression. *J. Biol. Chem.* **269:**3272–3276 (1994).
43. Farsetti, A., T. Mitsuhashi, B. Desvergne, J. Robbins, and V. M. Nikodem. Molecular basis of thyroid hormone regulation of myelin basic protein gene expression in rodent brain. *J. Biol. Chem.* **266:**23226–23232 (1991).
44. Baniahmad, A., C. Steiner, A. C. Kohne, and R. Renkawitz. Modular structure of a chicken lysozyme silencer: involvement of an unusual thyroid hormone receptor binding site. *Cell* **61:**505–514 (1990).

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