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A Novel Retinoid X Receptor-Independent Thyroid Hormone Response Element Is Present in the Human Type 1 Deiodinase Gene

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We identified two thyroid hormone response elements (TREs) in the 2.5-kb, 5′-flanking region of the human gene encoding type 1 iodothyronine deiodinase (*hdio1*), an enzyme which catalyses the activation of thyroxine to 3,5,3′-triiodothyronine (T3). Both TREs contribute equally to T3 induction of the homologous promoter in transient expression assays. The proximal TRE (TRE1), which is located at bp −100, has an unusual structure, a direct repeat of the octamer YYRGGTCA hexamer that is spaced by 10 bp. The pyrimidines in the −2 position relative to the core hexamer are both essential to function. In vitro binding studies of TRE1 showed no heterodimer formation with retinoid X receptor (RXR) β or JEG nuclear extracts (containing RXRα) and bacterially expressed chicken T3 receptor α1 (TRα) can occupy both half-sites although the 3′ half-site is dominant. T3 causes dissociation of TRα from the 5′ half-site but increases binding to the 3′ half-site. Binding of a second TR to TRE1 is minimally cooperative; however, no cooperativity was noted for a functional mutant in which the half-sites are separated by 15 bp, implying that TRs bind as independent monomers. Nonetheless, T3 still causes TR dissociation from the DR+15, indicating that dissociation occurs independently of TR-TR contact and that rebinding of a T3-TR complex to the 3′ half-site occurs because of its slightly higher affinity. A distal TRE (TRE2) is found at bp −700 and is a direct repeat of a PuGGTCA hexamer spaced by 4 bp. It has typical TR homodimer and TR-RXR heterodimer binding properties. The TRE1 of *hdio1* is the first example of a naturally occurring TRE consisting of two relatively independent octamer sequences which do not require the RXR family of proteins for function.

Type 1 iodothyronine deiodinase (D1), the product of the *dio1* gene, catalyzes both the first step in thyroid hormone activation, which is outer ring deiodination of thyroxine (T4) to form 3,5,3′-triiodothyronine (T3), and the inactivation of T4 or T3 by removal of an inner-ring iodine from the iodothyronine nucleus (48). In the rat, T3 produced by D1 activity provides ~60 to 70% of the circulating hormone (35, 52). D1 is one of a very small group of selenoenzymes in which selenocysteine, which is encoded by UGA, is present in the active center (8, 9). Thyroid hormone markedly increases *dio1* mRNA in liver and kidney in vivo and in rat FRTL-5 and pituitary tumor cells as well as in hepatocytes in primary culture (6, 7, 42, 47, 51, 67). This increase is a consequence of a direct interaction of T3 with the thyroid hormone receptor and does not require new protein synthesis as does the T3-induced increase in rat growth hormone (rGH) or spot 14 mRNA (59, 63).

Triiodothyronine induction of gene expression requires the interaction of ligand with the T3 receptor (TR), which is bound to specific sequences of DNA termed thyroid hormone response elements (TREs) and usually found in the 5′-flanking region of T3-responsive genes. A common TRE conformation consists of two core hexamers, termed half-sites, bearing a close resemblance to the consensus sequence PuGGTCA (15, 21, 37, 71). These half-sites may be found as direct repeats (DR) separated by 4 bp (DR+4), in a palindromic arrangement separated by 0 or 1 bp (PAL), or as everted palindromic repeats separated by 4 to 6 bp. When two such half-sites are present, they can bind two molecules of TR (homodimer) or heterodimeric complexes of TR with the retinoid X receptors (RXRs), retinoic acid receptor (RAR), peroxisomal proliferator activated receptor, chicken ovalbumin upstream promoter transcription factor, or other nuclear receptors (5, 14, 19, 20, 31, 38, 40, 44–46, 49, 69, 76, 77). Occupancy of the thyroid hormone receptor by T3 causes dissociation of TR homodimers from both DR+4 and everted palindromic repeats (but not palindromic arrangement separated by 0 or 1 bp) but has little effect on the binding of heterodimeric complexes containing RXR and TR (2, 19, 57, 70, 74, 75). Since two half-sites are generally required for TRE function, it is thought that heterodimeric complexes of T3-TR with other DNA-binding proteins such as the RXRs mediate the transcriptional effects of thyroid hormone on DR+4 or everted palindromic TREs (23, 39, 74).

Recent studies have pointed to potential exceptions to this concept. It is now recognized that nucleotides at positions −1 and −2 5′ to the consensus hexamer half-sites have a major influence on both the specificity and functional potency of a given TRE (15, 28–30, 60). Identification of an idealized TR-binding TRE by a PCR selection strategy (28, 29) and studies of systematic mutations of DR+4 TREs have shown that the preferred octamer binding site for conferring a positive response to T3 is YRRGGTCA (15, 60). A transcriptional response to T3 can be conferred by a high-affinity TR-binding octamer such as TAAGGTCA apparently without the cointeraction of a second TR or RXR (29, 60). To date, no naturally occurring TREs of this type have been identified.

The mouse *dio1* gene (*mdio1*) consists of four exons and a

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TATA-less promoter which is GC rich and contains an SP1-binding site (41). No TREs in the 5′-flanking region of that gene have yet been identified despite the fact that *mdio1* mRNA increases rapidly after T3 administration in vivo (41). In the present studies, we have evaluated the T3 response of the human *dio1* gene (*hdio1*). In HepG2 human hepatocellular carcinoma cells, *hdio1* mRNA is increased by T3 exposure. We have cloned the 5′-flanking region of *hdio1* and identified two TREs in the T3-responsive 2.5-kb, 5′-flanking region, both of which are located within 800 nucleotides of the transcription start site (TSS). A distal TRE, TRE2, has a DR+4 configuration of PuGGTCA hexamers and is found at bp −650 but contributes only about one-half of the T3 response. A more proximal response element, TRE1, is also necessary. TRE1 is a direct repeat of the YYRGGTCA octamer separated by 10 bp located on the antisense strand at bp −100.

The major goal of these studies was to characterize the mechanism of action of this unusual element. The pyrimidines in the first position of both octamers are essential to function. In contrast to classical TREs, in vitro DNA-binding studies showed no heterodimer formation on TRE1 between TR and RXRβ or JEG nuclear extracts which contain primarily RXRα (25, 65). Chicken TRα1 (cTRα) binds to both half-sites of TRE1, although the 3′ half site is dominant. The relatively large distance between the half-sites of TRE1 can be increased by 5 bp with only modest loss of function, suggesting that these widely spaced octamer half-sites function as monomeric TREs. The results presented herein show that TRE1 is the first example of a new class of TREs, direct repeat octamers which do not require interaction with RXR family proteins for function.

MATERIALS AND METHODS

HepG2 cell culture, RNA isolation, and Northern (RNA) blot analysis. HepG2, human hepatocellular carcinoma cells (32), were cultured in Dulbecco's modified Eagle's medium (GIBCO-BRL, Grand Island, N.Y.) supplemented with 10% fetal bovine serum. When the cells reached 80 to 90% confluence, the medium was replaced and then maintained in charcoal-stripped T3 and T4 free medium for 3 days (36). Thereafter, T3 (5×10^{-8} M) was added to the culture medium and incubated for 12 h. Where indicated, cycloheximide (3.5×10^{-5} M) was added 30 min before the T3. Total RNA was isolated by guanidinium-thiocyanate and cesium chloride gradients as described previously (43). Total RNA (30 μg) was separated on a 1.2% formaldehyde agarose gel and transferred to a nylon membrane (GeneScreen Plus; Dupont). The probe was a cRNA prepared from the human D1 (hD1) clone (43), which was transcribed in vitro by using T7 polymerase and [^{32}P]UTP as described previously (43).

Isolation and sequencing of *hdio1* genomic clones. A human placenta genomic library in λ-FixII vector (Stratagene, La Jolla, Calif.) was screened by hybridization with ^{32}P-labeled rat cDNA probe as described elsewhere (43). DNA from positive isolates was mapped after digestion with appropriate restriction enzymes by Southern hybridization. DNA fragments were subcloned into pBluescript (Stratagene) and were sequenced by the dideoxy chain termination method with Sequenase version 2.0 (U.S. Biochemical).

Primer extension. Total RNA from a thyroid tissue of a patient with Graves' disease was isolated by guanidinium thiocyanate and cesium chloride gradients as described previously (43). The 5′ end of the *hdio1* gene transcript was determined as previously described (4) by using a 35-bp oligonucleotide primer complementary to bp +66 to +100 of the hD1 cDNA (5′-CCACGACCACATGCA CAGCCACCTCCAAGAGCACC-3′) (see Fig. 1E) end labeled with [γ-^{32}P] ATP and T4 nucleotide kinase (Pharmacia). The primer (10^5 cpm) was hybridized with total RNA from human thyroid tissue or yeast tRNA at 42°C overnight and extended with Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories) for 1 h at 42°C. The resulting products were analyzed on a 6% polyacrylamide–8.3 M urea gel in parallel with a sequencing reaction generated with the extension primer.

Plasmid constructions. Standard techniques for DNA manipulation were used for all plasmid constructions (4). Genomic sequences from bp −716 to +26, −649 to +26, −248 to +26, −151 to +26, −107 to +26, and −99 to +26 were amplified by PCR with pBluescript containing the 2.3-kb *Eco*RI fragment (see Fig. 1B), together with forward and reverse primers. The forward primers contained a 5′-*Bam*HI adapter sequence to facilitate directional cloning. The reverse primer used was 5′-GCC**AG**ATCTCGGCAAAGCCAGAG-3′, corresponding to cDNA sequences from +31 to +9 except for mutations as indicated for introducing a *Bgl*II site and destroying the translation start codon ATG. PCR products were cloned into *Bam*HI site of plasmid pOCAT2, a promoter insertion chloramphenicol acetyltransferase (CAT) expression vector (56). These CAT constructs are designated HDCAT 716, HDCAT 649, HDCAT 248, HDCAT 151, HDCAT 107, and HDCAT 99, respectively. To generate 5′-to-3′ unidirectional deletion mutants, HDCAT 716 was cut with *Bam*HI and the following restriction enzymes: *Nco*I (−430) and *Xma*I (−81). The termini were blunt ended and ligated. These constructs are designated, respectively, HDCAT 430 and HDCAT 81. A *Bam*HI-*Xho*I 1.8-kb fragment from an *hdio1* genomic clone (see Fig. 1B) was ligated between the *Bam*HI and *Xho*I sites of the HDCAT 716 construct to make HDCAT 2.5 kb. Mutant HDCAT 716 (mHDCAT 716) was made by PCR mutagenesis (24). The reverse primer used was 5′-CCC**TTT** TCAGGGGAAGGAGTCA**TT**TCAGAGAAGATGTTTGCC-3′, corresponding to cDNA sequences from −79 to −129 with the exception of mutations (underlined) for destroying the consensus PuGGTCA half-sites. Synthetic double-stranded oligonucleotides for TRE1 and TRE2, shown in Fig. 2A and Fig. 3A, respectively, were inserted into the *Bam*HI site upstream of the thymidine kinase (TK) promoter in pUTKAT3 (56) as described previously (11). Oligonucleotides included sequences from the following genes: rat malic enzyme (ME; −287 to −260; 5′-AGGACGTTGGGGTTAGGGGAGGACAGTG-3′) and chicken lysozyme silencer F2 element (Lys F2; −2354 to −2326; 5′-TTAT TGACCCCAGCTGAGGTCAAGTTACG-3′). All constructs were sequenced by the dideoxy sequencing method.

Transient transfections and CAT assays. Transfections were carried out as previously described (11) with $CaPO_4$ precipitation in JEG, human embryonic kidney (HEK) 293, or COS 7 cells. The transfections were done in pairs, and each plate contained 10 μg of CAT reporter plasmid and 3 μg of TKGH (64) which constitutively expresses human GH (hGH). Transfections included 0.5 μg of CDM 8 vector (3) expressing mouse TRα1 (55) or rat TRβ1 (rTRβ1) (34) with or without a plasmid expressing human RXRα (hRXRα) (gift from David Mangelsdorf and Ronald Evans, Salk Institute, La Jolla, Calif.) or mouse RXRβ (mRXRβ) (gift from Keiko Ozato, NIH, Bethesda, Md.). CDM 8 was used to keep the amount of transfected DNA constant. The medium was made with charcoal-stripped sera with or without 5×10^{-8} M T3. CAT activity was determined by a phase extraction procedure modified from that described by Seed and Sheen (62) and was calculated as the percentage of conversion per 100 μl of cell extract protein in an overnight incubation (15 h), and the amount of hGH was determined as counts per minute per 100 μl of medium. T3 responsiveness (with T3 ÷ without T3) was expressed as the ratio of CAT to hGH from cells cultured in the presence of T3 divided by the CAT-to-hGH ratio from the respective paired plates cultured in the absence of T3. The transfection data are the means ± standard deviations (SD) of duplicate pairs from at least three separate transfection experiments.

Binding reactions and mobility shift assays. cTRα was overexpressed in *Escherichia coli* and purified as previously described (72). Gel shift experiments were performed under conditions previously described (72). The same probes used in the transient transfection constructions were radiolabeled with [^{32}P]deoxy-TTP (Dupont NEN, Boston, Mass.) by a Klenow fill-in reaction and were gel purified. Labeled probe (15,000 cpm; 4.4 fmol) was incubated with purified cTRα (2 to 16 fmol) in a 30-μl reaction mixture containing 100 ng of poly(dI-dC), 88 mM KCl, 10% glycerol, 25 mM Tris-Cl, 500 μM EDTA, 0.05% Triton X-100, 10 mM β-mercaptoethanol, and 5 μg of bovine serum albumin. Nuclear extracts from JEG cells were prepared as previously described (72) and were added as indicated. hRXRβ (a gift from Keiko Ozato, NIH, Bethesda, Md.) was made by in vitro translation and was added as indicated. Briefly, hRXRβ in pBluescript was linearized with *Hin*dIII and in vitro transcribed by using T3 polymerase and in vitro translated by using rabbit reticulocyte lysate (Promega, Madison, Wis.). A parallel translation reaction was run by using [^{35}S]methionine (Dupont NEN; >1,000 Ci/mmol) which yielded a translation product of the appropriate size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis. An amount of lysate equal to approximately 40,000 specific trichloroacetic acid-precipitable counts was added to each reaction mixture. Controls of equal amounts of H_2O-programmed lysate had no detectable RXR-like activity. These reaction mixtures were incubated for 30 min at room temperature and then analyzed at 4°C on a 5% nondenaturing polyacrylamide gel in low-ionic-strength buffer (10 mM Tris-Cl, 7.5 mM glacial acetic acid, 40 mM EDTA [pH 7.8]) and electrophoresed at 350 V. Antibody supershifting was performed with antibody to hRXRα (gift from David Mangelsdorf, Jacqueline Dyck, and Ronald Evans, Salk Institute). Supershifts were performed by allowing the complete binding reaction mixture to incubate for 20 min at room temperature and then adding antibody and incubating at 4°C for an additional 1 h. Competition experiments included 100 ng (1 pmol) of unlabeled competitor DNA.

Methylation interference assay. Methylation interference assays were performed as described previously (4). The pBluescript KS(+) plasmid containing the TRE1 oligonucleotide was cleaved with *Xho*I, and the antisense strand was 3′ end labeled with [^{32}P]dCTP by using the Klenow reaction before excision with *Sac*I. To end label the sense strand, the pUTKAT3 plasmid containing the TRE1 PCR fragment with the *Bam*HI site was cleaved with *Xba*I, labeled, and then excised with *Sac*I. The pUTKAT3 plasmid containing a TRE2 PCR fragment with the *Bam*HI site was cleaved with *Xba*I (sense) or *Xho*I (antisense), was end labeled, and then excised with *Xho*I (sense) or *Xba*I (antisense), respectively. End-labeled DNA fragments (100 ng) were then methylated with dimethyl sulfate as described previously (4). Preparative gel shifts were performed by using

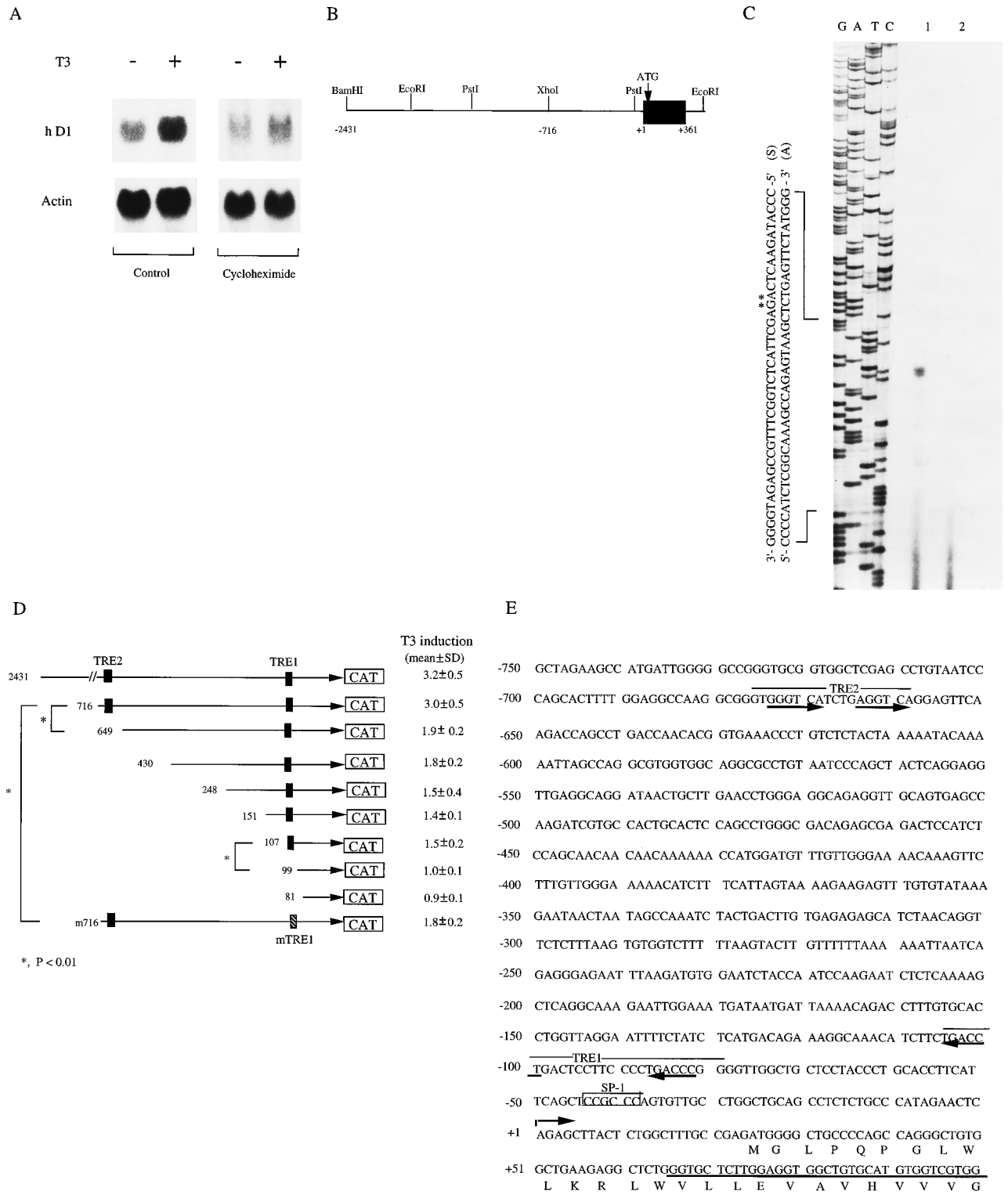


FIG. 1. (A) Northern analysis of the effect of T3 on *hdio1* mRNA expression in HepG2 cells. Total RNA (30 μg) from T3 (5×10^{-8} M)-treated (12 h) or untreated (with or without 30 min of cycloheximide pretreatment) HepG2 cells was run on a formaldehyde agarose gel and transferred to a nylon membrane. Blots were probed with ^{32}P-labeled cRNA for hD1. The same blots were hybridized with an actin probe as a control. (B) Schematic representation of the *hdio1* gene fragment containing the 5′-flanking region and exon 1 (black box). The translation initiation codon (ATG) is indicated, and the transcription start site is numbered +1. (C) Primer extension analysis. The transcription initiation sites were determined by primer extension analysis. The end-labeled 35-bp primer (see panel E) was hybridized to 70 μg of total RNA from thyroid tissue of a patient with Graves' disease (lane 1) and with 70 μg of *E. coli* tRNA as a control (lane 2). A sequencing ladder with the same primer was run in parallel. The nucleotides corresponding to the termination products are indicated by asterisks. S and A, sense- and antisense strand sequences, respectively. (D) T3 responsiveness of transient expression of various *hdio1*-CAT plasmids in HEK 293 cells cotransfected with an mTRα1 cDNA in a CDM expression vector and a TKhGH transfection efficiency control system. Constructs are numbered on the basis of the 5′ limit from the TSS. The T3 induction ratios (with T3-without T3) are expressed as the ratio of CAT to hGH from cells cultured in the presence of T3 divided by the CAT-to-hGH ratio from the respective paired cells cultured in the absence

purified cTRα with or without 100 nM T3 and 250,000 cpm of modified probe, and the specific binding and free probe bands were excised. The DNA was isolated, treated with 1 M piperidine for 30 min at 90°C, purified, lyophilized, and suspended in formamide loading buffer before being subjected to electrophoresis on an 8% denaturing polyacrylamide gel (4).

Quantitative analysis of Northern blots and receptor binding. Autoradiographs were quantitated by laser densitometry on a Molecular Dynamics model 3000 series computing densitometer (Molecular Dynamics, Sunnyvale, Calif.), by using Molecular Dynamics Image Quant software. Densitometry was performed on several exposures for each gel to determine that the band density was in a linear range.

Statistical analysis. Statistical analysis was performed by using StatView 4.0 (Abacus Concepts, Berkeley, Calif.). The significance of differences in T3 induction between TRE1 and each TRE1 mutation was confirmed by one-way analysis of variance and then by Scheffe's test for pairwise comparisons.

RESULTS

***hdio1* is regulated by T3 via transcriptional stimulation.** *hdio1* mRNA is expressed in HepG2 cells, and the levels increase two- to threefold after 12 h of exposure to T3 (Fig. 1A). As is the case in rat thyroid, liver, and pituitary tumor cells, the stimulatory effect of T3 is not inhibited by preexposure of the cells to cycloheximide (Fig. 1A). A clone containing the *hdio1* 5′-flanking region was obtained from a human genomic library by probing with a hD1 cDNA (Fig. 1B). The TSS was identified by primer extension and S1 nuclease mapping by using total RNA isolated from thyroid tissue from a patient with Graves' disease. The primer extension products terminated at two consecutive nucleotides, C and T, 23 or 24 bp upstream from the translation initiation codon (Fig. 1C). S1 nuclease mapping showed one major protected band with a size consistent with initiation at the same position indicated by the primer extension assay (data not shown).

A 2.5-kbp fragment of the 5′-flanking and 5′-untranslated region was sequenced and subcloned into pOCAT2. When the pOCAT2 vector containing the 2.5-kb fragment was transfected into HEK 293 cells together with a eukaryotic expression vector containing a cDNA encoding the mTRα1 cDNA (mTRα1), T3 induced a threefold increase in CAT expression with mTRα1, which is comparable to the transcriptional response in HepG2 cells and a twofold response with rTRβ1 (Fig. 1D). Deletion studies identified two positive thyroid hormone regulatory regions in this fragment, one of which is between −107 and −80 (TRE1) and the other of which is between −716 and −649 (TRE2) (Fig. 1D). The sequence of this portion of the promoter and 5′ flanking region showed two putative TREs in these areas: a direct repeat of the RGGTCA TR binding consensus hexamer with a 4-bp gap (DR+4) in the upstream region (TRE2) and a second direct repeat of an octamer YYRGGTCA on the antisense strand with 10 bp separating the two half-sites (octamer DR+10) in the promoter (TRE1) (Fig. 1E). Mutation of the core G residues of the putative TRE1 half-sites (mHDCAT 716) reduced T3 induction to 1.8-fold ($P < 0.01$), indicating that both TREs are essential for the full T3 response of the *hdio1* promoter (Fig. 1D). We first turned our attention to the study of TRE1, although, for certain analyses, TRE2 is included as an example of a classical DR+4 TRE.

TRE1 has a unique structure. TRE1 consists of two octamer consensus half-site motifs separated by 10 bp. The most 5′ nucleotide of both octamers is a pyrimidine corresponding to an optimal TRE; however, those at the second positions are not the preferred purines. This TRE is essential for a full T3 response with the homologous promoter (Fig. 1D) and conferred a 2.3-fold induction to the TK promoter (Fig. 2A). Since apo-TR (unliganded) did not reduce basal expression of TRE1-TKCAT (although it did reduce the 716-bp *hdio1* promoter expression), the positive response is due to an absolute increase in TK promoter activity. A mutant TRE in which the five G's in the spacer region were changed to T's (mutation 22) conferred the same T3 response as did the wild-type TRE1, indicating that these residues are not cryptic binding sites required for its function (Fig. 2A). Mutation of either or both of the 5′ pyrimidines to purines (mutations 23, 24, and 25) reduced or eliminated function ($P < 0.01$). In addition, G-to-T substitutions in the core G residues of either half-site (mutations 26 and 27) eliminated the function of TRE1 ($P < 0.001$). Similar results were obtained with COS 7 and HEK 293 cells (data not shown). The results of these experiments confirmed that the function of this novel TRE required specific nucleotides in both octamer half-sites.

Biochemical studies indicated that single and double occupancy of TRE1 was a function of cTRα concentration (Fig. 2B). Surprisingly, the affinity of TRE1 for TR was comparable to that of the well-studied ME TRE, which has a DR+4 configuration; however, both bind TR less avidly than does TRE2 (Fig. 2B and C). The differences between TRE1 and TRE2 parallel their differential function with respect to the TK promoter (see below); however, ME confers a T3 response to the TK promoter that is three- to fourfold greater than that of TRE1 in the same experiments. Cooperativity plots (68) showed a correlation between the binding of a second TR and TRE function with ratios of double to single occupancy of both TRE2 and ME that were consistently higher than that of TRE1 (Fig. 2C). Mutation of the core GG dinucleotide of the 3′ half-site (mutation 26) markedly reduced the affinity for TRα binding (Fig. 2D and E). The comparable mutation of the three G residues in the 5′ half-site (mutation 27) reduced double occupancy only slightly (Fig. 2D and E), although both mutations were nonfunctional. A Y-to-R mutation of nucleotide 1 of the 3′ half-site (mutation 24) reduced double occupancy by TR substantially, while the comparable change in the 5′ site (mutation 25) had no effect on TR binding. Despite the normal TR binding to mutation 25 (Fig. 2D and E), it was also inactive.

TRE1 does not form heterodimers with RXRs or nuclear proteins of JEG cells, nor does RXR enhance T3 induction. Formation of TR heterodimers with RXR, RAR, or other nuclear proteins is a property common to all naturally occurring TREs reported to date. No binding of TRE1 or the ME TRE is found with JEG nuclear extracts (Fig. 3A, lanes 1 to 3 and 6 to 8). Surprisingly, TRE1 also does not form heterodimers with proteins in JEG cell nuclear extracts (Fig. 3A, lanes 9 and 10), even though heterodimer formation is quite apparent on the ME TRE when the same preparations are used (Fig. 3A, lanes 4 and 5). A supershift by anti-hRXRα (lane 5) corresponding to the major RXR protein in JEG cells (25, 65) confirms the presence of an RXRα-TR-TRE low-mobility complex in lane 4. No low-mobility complex is found

of T3. The TREs are depicted by black boxes. The mutant TRE1 (mTRE1) prepared by PCR as described in Materials and Methods is depicted by a shaded box. The transfection data are the means ± SD of duplicate pairs of plates from at least three separate transfection experiments. Statistical significance was evaluated by Student's *t* test. (E) Nucleotide sequence of the 5′-flanking region of *hdio1* including a 5′ portion of exon 1. The nucleotide sequence is numbered, with +1 referring to the major TSS, and nucleotides upstream are assigned negative numbers. The recognition site for Sp1 is boxed. Putative TRE half-sites are denoted by horizontal arrows. A sequence complementary to that underlined in the coding region was used as a primer in the primer extension analysis.

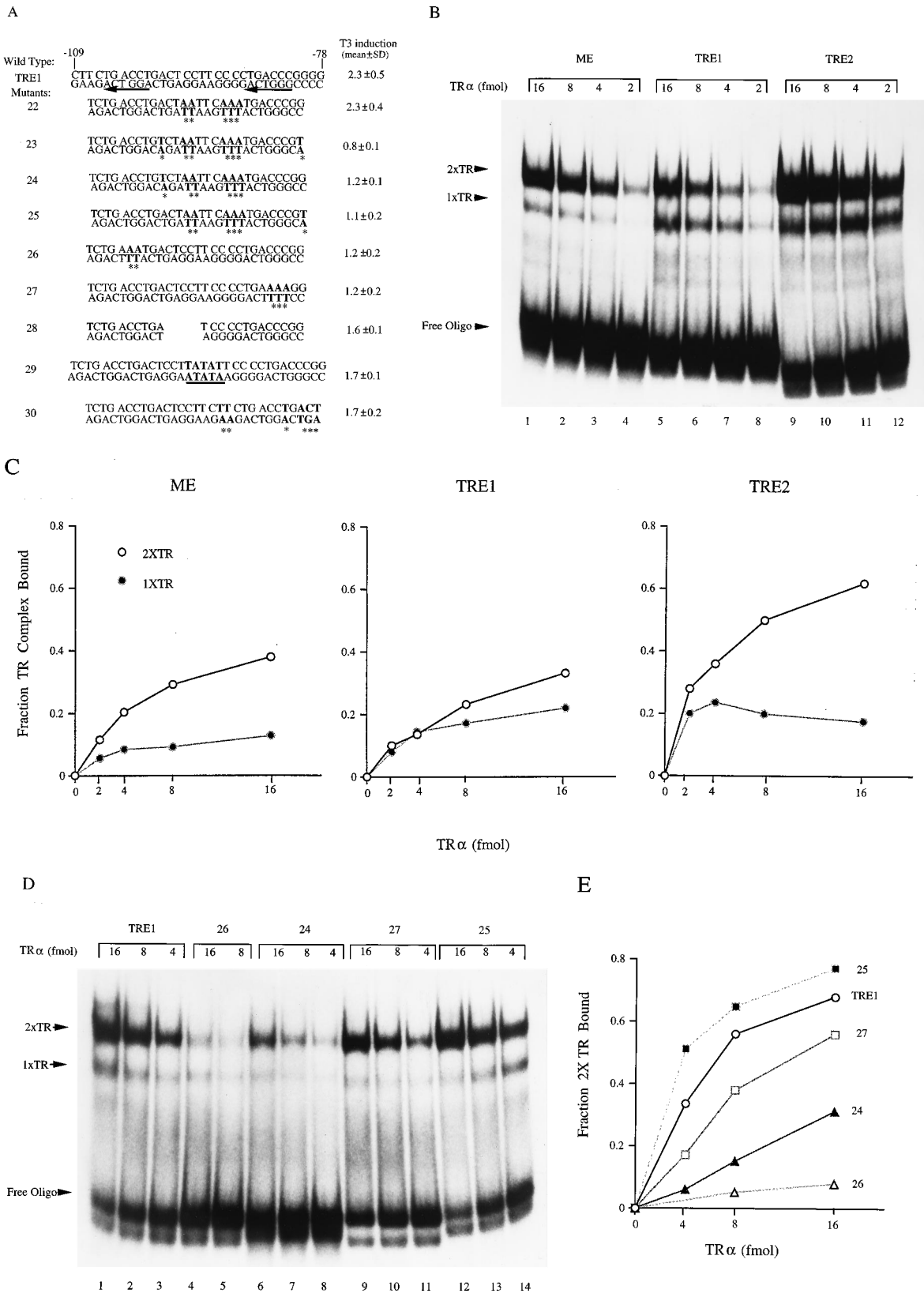


FIG. 2. (A) Functional analysis of wild-type and mutant TRE1 T3 response elements in JEG cell transient transfections. Sequences of oligonucleotides inserted into the pUTKAT vector to test T3 response in transient transfection studies are shown. The means ± SD for the expression of these constructs transiently transfected into JEG cells together with CDM mTRα1 and TKhGH are given. Values are the ratios of the induction of TRE-TKCAT (with T3 ÷ without T3) construct versus those of TK alone (with T3 ÷ without T3) in the same transfection. The T3 induction of the TKCAT vector (CAT ÷ hGH) is 1.6 ± 0.3-fold in JEG 3 cells under these conditions. The arrows in the wild-type and mutant elements indicate RGGTCA half-sites. The mutations are indicated by asterisks. (B) Binding of purified cTRα to ME TRE

(ME), TRE1, and TRE2. The ^{32}P-labeled ME, TRE1, and TRE2 oligonucleotides (oligo) (15,000 cpm [4.5 fmol] each) were incubated with increasing amounts of purified cTRα (2 to 16 fmol), and protein-DNA complexes were separated on polyacrylamide gels. 1× and 2×, single and double binding, respectively. (C) Comparison of double and single TR binding to ME, TRE1, and TRE2. Double (2×TR) and single (1×TR) occupancy was quantitated by densitometry from the gels shown in panel B. (D) Binding of purified cTRα to TRE1 and mutant elements. The ^{32}P-labeled ME, TRE1, and mutant element (nos. 24, 25, 26, and 27) oligonucleotides (15,000 cpm [4.5 fmol] each) were incubated with increasing amounts of purified cTRα (2 to 16 fmol), and protein-DNA complexes were separated on polyacrylamide gels. (E) Effect of 3′ (mutant elements 24 and 26) and 5′ (mutant elements 25 and 27) octamer half-site mutations on double and single occupancy by TRα. Sequences are shown in Fig. 3A. Double TR occupancy (2×TR) was quantitated by densitometry from the gel shown in panel D, and the amounts of 2×TR bound are plotted. Results are typical of several similar experiments.

with TRE1 and no supershifted complex is found with anti-RXRα antibody (Fig. 4A, lanes 9 and 10).

Similar experiments were performed with in vitro-translated hRXRβ, with similar conclusions (Fig. 3B). The Lys F2 element used as a control in these studies bound both TR homodimeric and in vitro-translated RXRβ-TR heterodimeric complexes. Thyroid hormone caused partial dissociation of the TR homodimer (Fig. 3B, lanes 2 and 4) and also caused an increase in the level of T3-TR monomer binding (lanes 1 and 3 versus 2 and 4). In contrast, no heterodimer with TRE1 was found in the absence or presence of T3 (Fig. 3B, lanes 10 and 11). In the presence of unprogrammed reticulocyte lysate (Fig. 3B, lanes 7 and 14), single and double occupancy by TR was enhanced presumably because of nonspecific effects of lysate proteins, but there was no RXR-like activity. A similar effect in lane 11 thus cannot be ascribed to the presence of RXRβ. Note the marked reduction of double TR occupancy of TRE1 by 100 nM T3 (Fig. 3B, lane 8 versus 9 and 10 versus 11).

The effects of RXRα and RXRβ on TRE function were investigated by studying the effects of cotransfection of CDM vectors expressing hRXRα or mRXRβ with TR into COS 7 cells. The T3 induction ratio of TKCAT was increased from 1.9 to 3.9 by cotransfection of an RXRα expression plasmid, presumably because of the fact that this promoter contains a weak everted palindromic TRE (53). CAT expression from TRE1 TKCAT was increased in a parallel fashion, with T3 induction increasing from 6.1 in the absence of RXRα to 12.2 when RXRα cDNA was cotransfected. In contrast, RXRβ cotransfection did not alter the T3 stimulation of TKCAT, TRE1 TKCAT, or the 107-bp *hdio1* CAT construct which contains TRE1 (data not shown).

Methylation of guanines of the 3′ and 5′ half-sites on both the sense and antisense strands interfered with double-occupancy TR binding (Fig. 4A). G's in the 3′ half-site were more important contact sites than those in the 5′ half-site, as judged by the lower density of bands corresponding to these G residues. In the fragment binding only a single TR, only the 3′ half-site was protected, indicating that the occupancy of this half-site is preferred. Addition of T3 caused a significant reduction or elimination of double occupancy by TR (Fig. 4B). Fragments containing methylated G residues in the fragment-binding T3-TR monomer were decreased in the 3′ half-site but not in the 5′ half-site (Fig. 4B). Thus, in both the presence and the absence of T3, TR binding to the 3′ half-site is preferred.

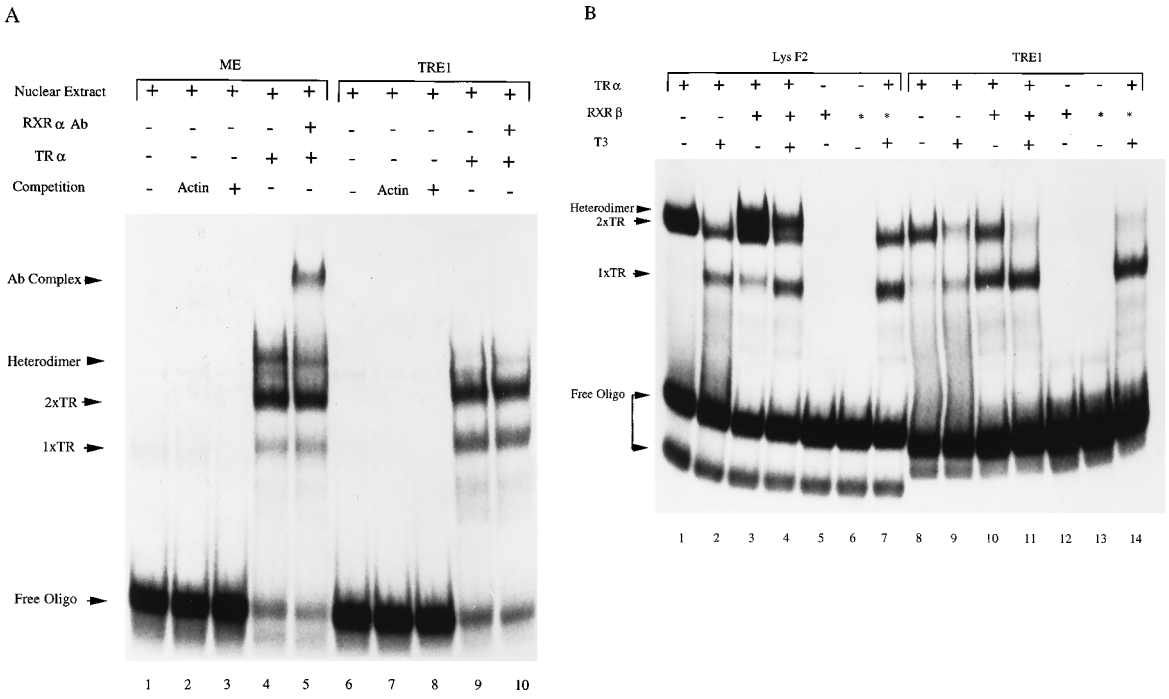


FIG. 3. (A) Gel shift assay with nuclear extract from JEG cells. Labeled TRE1 and ME TRE (ME) probes (15,000 cpm [4.5 fmol] each) were incubated with cTRα (8 fmol). Each binding reaction mixture included 4 μg of nuclear extract protein from JEG cells. Competition was with either β-actin DNA (100 ng) (lanes 2 and 7), ME DNA (100 ng) (lane 3), or TRE1 DNA (100 ng) (lane 8). Antibody (Ab) (1.5 μl) to hRXRα was added (lanes 5 and 10) to supershift TR-RXR heterodimer complexes. Oligo, oligonucleotide. (B) Gel shift assay with in vitro-translated RXRβ. Labeled TRE1 and Lys F2 probes (15,000 cpm [4.5 fmol] each) were incubated with 2 fmol (Lys F2) or 8 fmol (TRE1) of purified cTRα, respectively, with or without in vitro-translated RXRβ in the presence or absence of T3 (100 nM). Equal amounts of H_2O programmed lysate were added (lanes 6, 7, 13, and 14) as a control (indicated by asterisks). 1× and 2×, single and double binding, respectively.

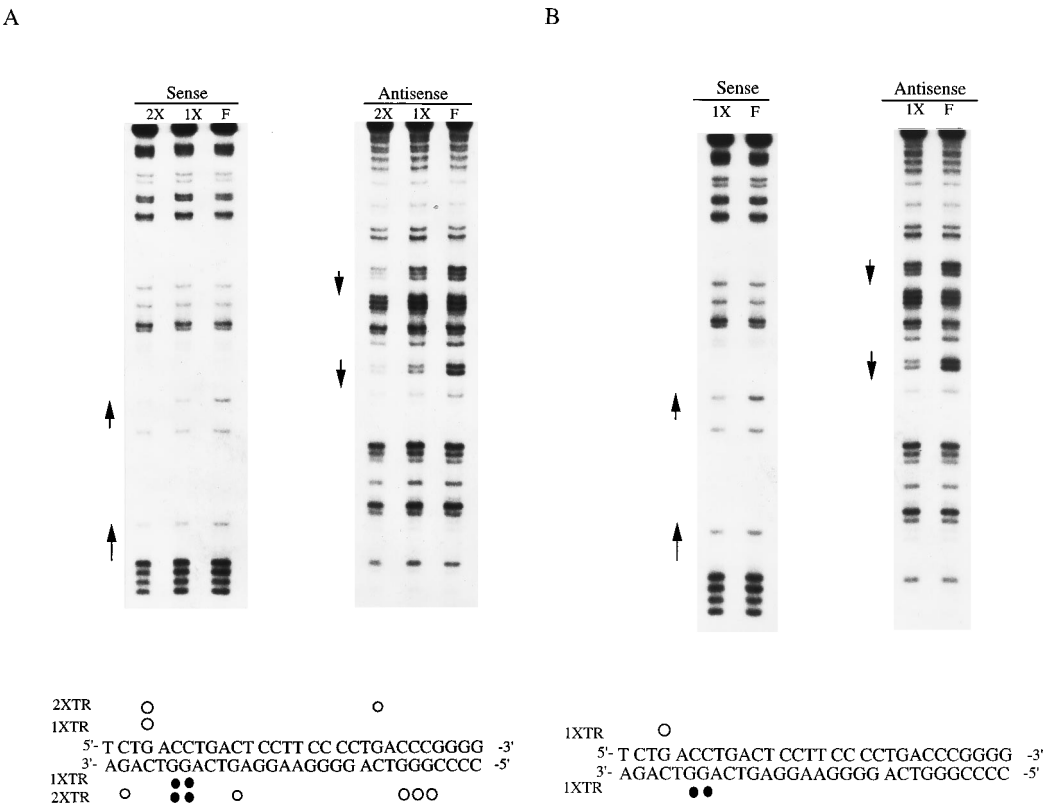


FIG. 4. Methylation interference footprinting of TRE1 by TR in the absence and presence of T3. Methylation interference was performed as described in Materials and Methods. The DNA fragment containing TRE1 was selectively labeled on either the sense or the antisense strand and methylated with dimethyl sulfate. The methylated DNA fragments were incubated with purified cTRα in the absence (A) or presence (B) of T3 (100 nM), and protein-DNA complexes were separated on nondenaturing polyacrylamide gels. Probe with both sites (2×TR) or one site (1×TR) occupied by TR and free (F) probe was recovered, cleaved with piperidine, and analyzed on sequencing gels. Arrows, the positions of the two half-sites; circles, positions of major groove interference caused by N7 methylation of guanine; solid symbols, complete or nearly complete interference; open symbols, weak interference (25 to 50% of the intensity of the corresponding band in free probe lanes).

Altering the separation between half-sites has minimal effects on function but alters the cooperativity of TR binding. The large separation between the two half-sites of TRE1, its most unique aspect, suggested that the level of interaction between TRs bound to these two half-sites would be low. This is confirmed by a low degree of cooperativity for TR double occupancy on this fragment compared with the ME TRE or TRE2 (Fig. 2C). To explore the role of spacing on both function and TR binding, we prepared mutations 28 (DR+5) and 29 (DR+15) (Fig. 2A). Decreasing or increasing the distance between the two binding sites by 5 bp (one-half turn of the α-helix) modestly decreased function ($P < 0.01$ for DR+5 and $P < 0.02$ for DR+15 [Fig. 2A]). Occupancy by a single TR or T3-TR complex was also only slightly reduced by altered spacing (Fig. 5A and B). However, the degree of cooperativity between the binding of monomer and a second TR was modest for the wild type (DR+10) and enhanced for mutation 28 (DR+5) but was eliminated for mutation 29 (DR+15) (Fig. 5B). The effect of T3 on TR binding was similar for all three TREs, in that double occupancy was markedly reduced and T3-TR monomer binding increased (Fig. 5C). Analysis of TR affinity by densitometry confirmed that TR-T3 monomers bound equally well to all three DNA fragments (data not shown).

Affinity of the half-sites of TRE1 determines the degree of double occupancy by liganded TR. To explore the role of half-site sequence on TR binding, we investigated the binding and function of mutation 30, in which the 13 bp encompassing the 3′ half-site was repeated at the 5′ site (Fig. 2A). Surprisingly, double or single occupancy of this mutant by apo-TR was not altered by this change (Fig. 6). However, the affinity of mutation 30 for double occupancy by T3-TR was 150% that of TRE1, indicating that when the affinity of the 5′ half-site is the same as that of the 3′ half-site, T3-TR binding increases. Interestingly, the function of mutation 30 was slightly decreased by this modification (Fig. 2A).

TRE2 is a classical TRE of the DR+4 class. TRE2 confers a ninefold T3 response to the TK promoter when cotransfected with mTRα1 in JEG cells (Fig. 7A). apo-TR did not reduce basal CAT expression with TRE2, with the result that stimulation reflected only the increase in the TRE2-TK promoter activity by T3-TR. Mutant TREs containing G-to-T mutations of either the 3′ half-site (mutation 11) or 5′ half-site (mutation 12) did not confer a T3 response under the same conditions. The 3′ half-site of TRE2 is preceded by a YR dinucleotide, thus corresponding to an optimal TR-binding octamer. A T-to-A change at the −2 position of the 3′ half site (mutation 10) reduced T3 induction by 20% ($P < 0.01$) (Fig. 2A). Gel mobility shift assays with *E. coli*-expressed cTRα1 with or without in vitro-translated hRXRβ showed that TRE2 could bind either homo- or heterodimers (Fig. 7B). In the presence of T3, heterodimer formation was not altered, homodimer binding was markedly reduced, and T3-TR monomer binding was increased. The mobilities of both liganded monomer and heterodimer bands were increased. The pattern of homo- and heterodimer binding on mutation 10 was similar, although

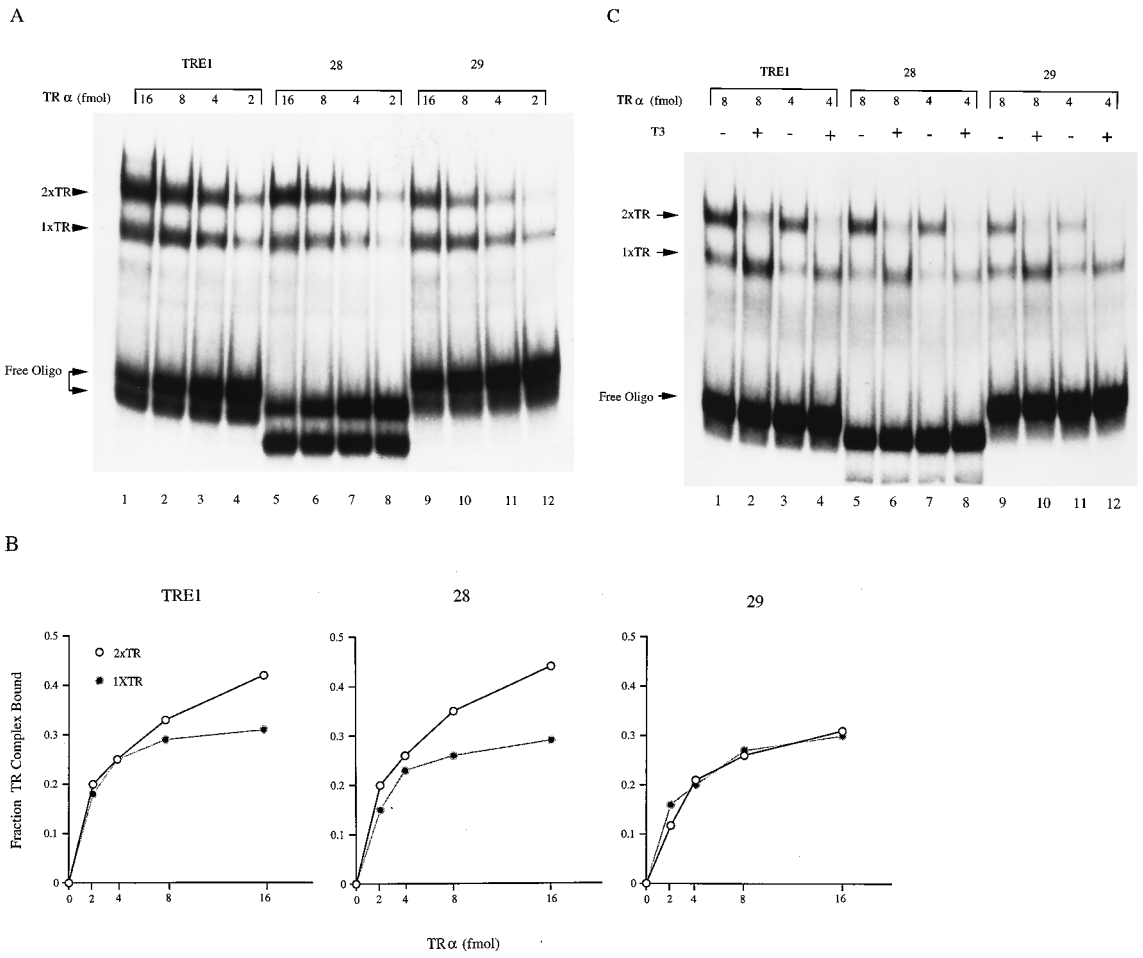


FIG. 5. (A) Binding of purified cTRα to TRE1 and mutant elements. The ^{32}P-labeled TRE1 and mutated element (nos. 28 and 29) oligonucleotides (15,000 cpm [4.5 fmol] each) were incubated with increasing amounts of purified cTRα (2 to 16 fmol), and protein-DNA complexes were separated on a polyacrylamide gel. The gel represents several experiments. (B) Double (2×TR) and single (1×TR) TR occupancy was quantitated by densitometry from gels shown in panel A, and the amounts of 2×TR or 1×TR bound are plotted. (C) Binding of liganded cTRα to TRE1 and mutant elements. The ^{32}P-labeled TRE1 and mutated element (nos. 28 and 29) oligonucleotides (15,000 cpm [4.5 fmol] each) were incubated with purified cTRα (4 or 8 fmol) in the presence or absence of T3 (100 nM), and protein-DNA complexes were separated on a polyacrylamide gel.

T3-TR monomer binding was virtually eliminated because of the T-to-A mutation at the −2 position of the 3′ half-site. This is consistent with methylation interference footprinting results indicating that the T3-TR monomer preferentially binds to the 3′ half-site (Fig. 7C). No binding of TR or RXR on mutation 11 (the 3′ half-site mutation) was observed, indicating a marked decrease in its affinity by mutation of the dominant 3′ half-site. On the other hand, mutation 12 (the 5′ half-site mutation) bound TR and T3-TR monomers with the same affinity as TRE2 but bound hetero- or homodimers much less avidly (Fig. 7B).

To identify the contact sites on TRE2 for TRα complexes, we performed methylation interference studies (Fig. 7C). The TRα homodimer strongly contacted the core G residues of the 3′ half-site on the sense strand and the 3′ G residue of the antisense strand. Weak contacts were also made with the G residues at positions −1 and −3 of the antisense strand of this half-site. Weak interaction was present with the three core G residues (sense strand) and with the 3′ G residues of the antisense strand of the 5′ half-site. The TRα monomer contacted only the G residues of the 3′ half-site.

DISCUSSION

Characteristics of the *hdio1* promoter. The *hdio1* promoter does not contain a TATA box but has an SP1 binding site 40 bp upstream of the TSS, as is typical of TATA-less promoters (Fig. 1E). An initiator sequence (INR) is present at the start site with a conserved CA at the −1 and +1 positions, and the region 5′ to the INR is pyrimidine rich, as is typical of the INR consensus sequence, although the preferred T at position +3 is not present (27). The INR sequences of the mouse and human genes are identical, and *mdio1* also has the SP1-binding site. The human and mouse promoters have 72% identity over the 260 bp 5′ to the TSS (41). The *hdio1* mRNA is T3 responsive in HepG2 cells and the increase of both rat *dio1* and *hdio1* mRNAs is not blocked by 30 min of preincubation with cycloheximide, indicating that this response does not require protein synthesis or the presence of short-lived proteins. The T3 responsiveness of the 2.5-kb promoter and 5′-flanking region can be explained by the presence of two distinct TREs which contribute equally to the T3 induction of the homologous promoter (Fig. 1D). The threefold response to T3 of the *hdio1*

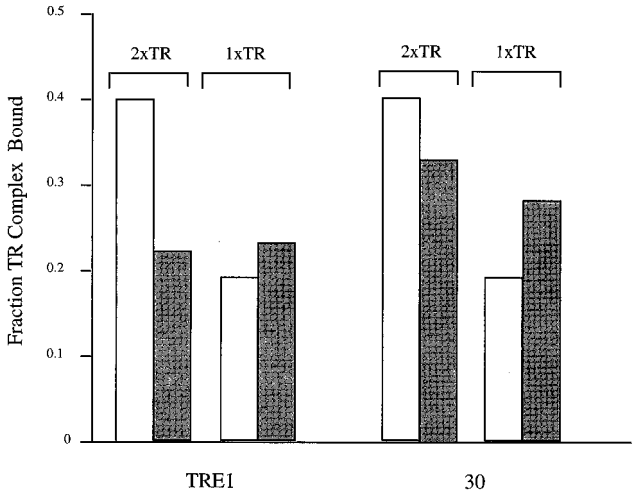


FIG. 6. Binding of cTRα to TRE1 and mutant element 30. The ^{32}P-labeled TRE1 and mutated element 30 oligonucleotides (15,000 cpm [4.5 fmol] each) were incubated with purified cTRα (8 fmol) in the presence (solid columns) or absence (open columns) of T3 (100 nM), and protein-DNA complexes were separated on a polyacrylamide gel. Double (2×TR) and single (1×TR) TR occupancy was quantitated by densitometry. The results represent several experiments.

promoter and 5′-flanking region is modest; however, in transient expression assays it is slightly higher than that which occurs with the well-studied rGH gene (36). It is consistent with the magnitude of the acute induction of the endogenous gene in the HepG2 cells (Fig. 1A).

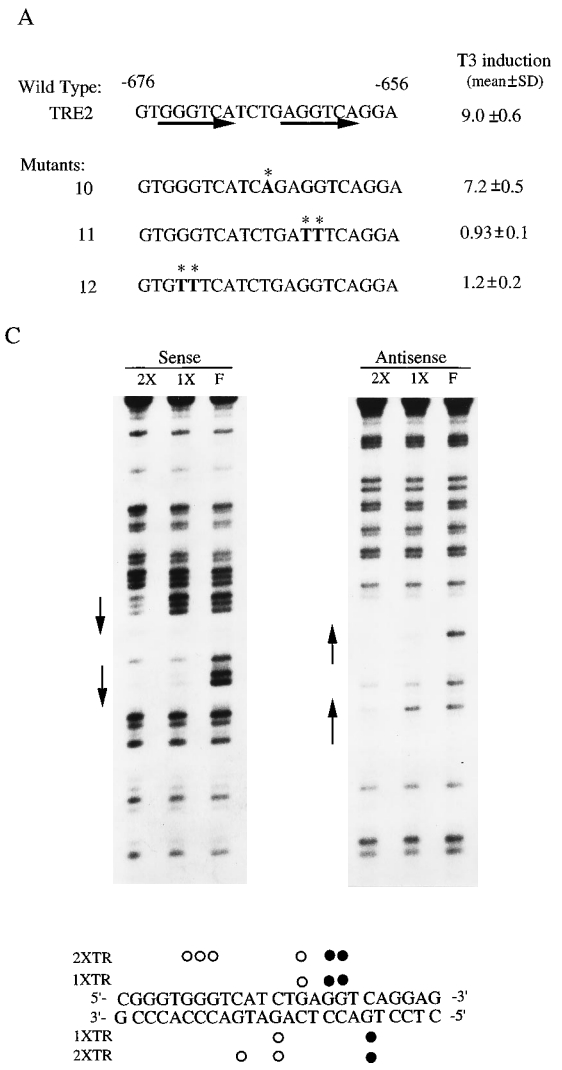
TRE1 has a novel DR+10 conformation. The proximal *hdio1* TRE is a direct repeat of a consensus octamer with a 10-bp separation, which is an arrangement which has not been described previously. The only naturally occurring TRE previously reported, with more than 4 bp separating the two half-sites, is in the long terminal repeat of the human immunodeficiency virus type 1 (HIV-1) promoter. This TRE colocalizes with NF-κB binding sites (18). While footprinting results have not been reported, the HIV-1 sequence is compatible with either 8- or 10-bp spacing between the half-sites. While the response conferred by a single copy of TRE1 to homologous or heterologous promoters is not of high magnitude, it is consistent and sufficiently high to permit interpretable mutational studies (Fig. 1 and 2). Each half-site of TRE1 contains a pyrimidine at the −2 position, both of which are required for function (Fig. 2A). Several reports have illustrated the critical role of the two nucleotides 5′ of the hexamer binding site in TR binding and function (15, 28–30, 60). The most striking example is the monomer TAAGGTCA, which has been selected from a series of random oligomers for TR binding by a PCR strategy and is sufficiently potent to confer a thyroid hormone response without a requirement for RXR (28, 29). Systematic studies of the −1 and −2 nucleotides 5′ of the consensus hexamer have shown that the idealized DR+4 hexamer contains a pyrimidine at the −2 position and prefers a purine at position −1, although the latter is not essential and is not present in TRE1 (60). Methylation footprinting shows that both half-sites of TRE1 can be occupied by TRα but that a single TR is preferentially bound to the 3′ half-site (Fig. 4A). While the 3′ half-site in many conventional DR+4 and everted repeat TREs is dominant, this has been shown in one instance to be a consequence of a higher intrinsic affinity of the 3′ half-site sequence rather than its 3′ position in the TRE (26). Consistent with a similar explanation for TRE1 is our observation that the affinity of TRE1 for double occupancy by liganded TRs is increased by making the 5′ half-site identical in sequence to the 3′ half-site (Fig. 6). The only differences between the half-site sequences are found at positions 1 and 3 of the octamer (Fig. 2A). Preferential binding of a TR monomer to a TGAGGTCA octamer (3′ half-site) as opposed to a TGGGGTCA octamer (5′ half-site) with the F2 element has also been noted (26). This suggests a preference for adenine in this position for TR binding, although a G-to-A change in the 5′ half-site of F2 has little effect on functional potency (25).

Another unique feature of TRE1 is that, unlike previously described TREs including the monomer element (29), neither RXRα nor RXRβ binds as a heterodimer with TR (Fig. 3A and B) and the cotransfection of RXRα- or RXRβ-expressing plasmids does not specifically enhance the response of TRE1 TKCAT plasmids to thyroid hormone, although the response to T3 of the TKCAT plasmid itself is increased via an unknown mechanism. The enhancement of the TKCAT response to T3 could occur through heterodimer formation on the weak TRE previously identified in the TK promoter (53). The characteristics of TRE1 are thus quite distinct from those described for the TRE- and/or NF-κB-binding sites in the HIV-1 promoter which binds RXRβ-TR heterodimers (18).

The predominance of TR occupancy of the 3′ half-site for both liganded and apo-TR and the loss of TR binding when this site is mutated (Fig. 2E) indicate that the downstream octamer has a higher affinity for TR than does the 5′ half-site. On the other hand, the low degree of cooperativity of TR binding on TRE1 (Fig. 2C and 5B) suggests that these two half-sites bind TR relatively independently. Altering the distance between the half-sites by a half-turn of the α-helix slightly reduced their function relative to that of the native element (Fig. 2A), but there was no apparent decrease in the capacity for double occupancy by TR (Fig. 5C). However, there was a consistent effect on the cooperativity of TR binding in that reducing the spacing by 5 bp increased it, whereas the curves of single and double occupancy of mutation 29 were superimposable (Fig. 5B). The cooperativity estimate for TRE1 was intermediate between the two. The cooperativity for mutation 28 is comparable to that of the wild-type DR+4 TRE class in ME (Fig. 2C).

The results of these studies argue that there is a modest cooperativity of TR binding to TRE1 despite the 10-bp separation between the half-sites. The biochemical role of TR binding to the 3′ half-site is reflected in the marked decrease of the affinity of the TRE for apo-TR when G-to-T mutations are made in the core G residues or a Y-to-R mutation is made at the first position of the 3′ half-site (Fig. 2D and E). These results suggest that binding of a TR monomer to the 3′ half-site precedes that to the 5′ half-site, which is consistent with the methylation footprinting results. Once the 3′ half-site is occupied, the binding of a second TR is slightly favored over TR dissociation from it. Presumably, a weak interaction between the first and second TR occurs between portions of the ligand-binding domain, since the distance is too great for interaction in the putative homodimerization domain of the D box of the second zinc finger (20, 21). Increasing the spacing between the half-sites to 15 bp eliminates this weak interaction because of either the greater distance, the angular rotation of the half-sites, or both.

Dissociation of homodimer TR binding by ligand is a consistent observation for both the everted palindromic chicken Lys F2 and DR+4 elements, although it does not occur on a palindromic TRE (2, 19, 57, 70, 73, 75). Our results provide evidence that this phenomenon also occurs on an element in



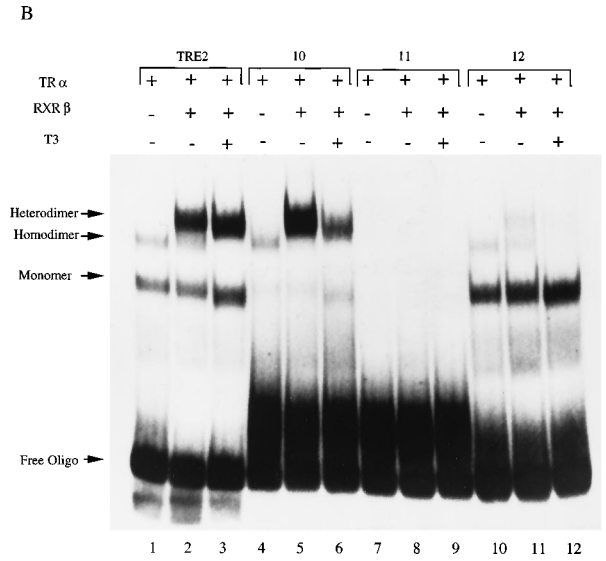


FIG. 7. (A) Functional analysis of wild-type and mutant element TRE2 T3 response in JEG cell transient transfections. The sequences of oligonucleotides inserted into the pUTKAT vector to test the T3 response in transient transfection studies are shown. The means ± SD are shown for the expression of these constructs transiently transfected into JEG cells together with CDM mTRα1 and TKhGH. Values are the ratios of the induction of the TRE-TKCAT construct (with or without T3) versus those of TK alone (with or without T3). The induction of the TKCAT vector (CAT-hGH) is 1.6 ± 0.3-fold in JEG 3 cells under these conditions. The arrows in the wild-type and mutant elements indicate RGGTCA half-sites. The mutations are indicated by asterisks. (B) Binding of purified cTRα to wild-type TRE2 and mutated elements. Gel mobility shift assays were used to study the binding of 2 fmol of purified cTRα with ^{32}P-labeled TRE2 and the respective mutated elements (15,000 cpm [4.5 fmol] each), in the presence or absence of in vitro-translated RXRβ and/or 100 nM T3. Oligo, oligonucleotide. (C) Methylation footprinting of TRα binding to TRE2. Methylation interference was performed as described in Materials and Methods. The DNA fragment containing TRE2 was selectively labeled on either the sense or antisense strand and methylated with dimethyl sulfate. The methylated DNA fragments were incubated with purified cTRα, and protein-DNA complexes were separated on nondenaturing polyacrylamide gels. Retarded probes with two sites (2×TR) or one site (1×TR) occupied by TR and free (F) probe were recovered, cleaved with piperidine, and analyzed on sequencing gels. Arrows, the positions of the two recognition motifs; circles, positions of major groove interference caused by N7 methylation of guanine; solid symbols, complete or nearly complete interference; open symbols, weak interference (25 to 50% of the intensity of corresponding bands in free lanes).

which nonconventional interactions between the two TRs are present. As with DR+4 TREs, the binding of the monomeric T3-TR complex is preferentially to the 3′ half-site of TRE1. Dissociation of one of the TRs by T3 also occurs on the spacing mutation 29. Given the absence of cooperativity of TR binding for mutation 29 and the methylation footprinting studies (Fig. 4), monomeric T3-TR binding must occur on the 3′ half-site because of the greater affinity for T3-TR of the AGGTCA core hexamer compared with that of the GGGTCA core hexamer. Studies of liganded-TR binding to DNA have suggested that the affinity of T3-TR is reduced two- to threefold over that of apo-TR, and circular dichroism results have illustrated a marked change in the conformation of the receptor when a ligand occupies its binding site (2, 66, 70). In gel shifts, this is reflected in the more rapid migration of the DNA containing a T3-TR monomer, which was also seen in our experiments. The higher double occupancy by T3-TR on mutation 30 than on TRE1 (Fig. 6) confirms this hypothesis and argues against steric hindrance as an explanation for the failure of two liganded TRs to bind to TRE1.

A surprising result was that certain mutations in TRE1 reduce its function without causing detectable changes in DNA binding. For example, compare the apparently normal TR binding affinity of mutations 25 and 27 (Fig. 2D and E) with the inability of these mutants to confer a T3 response (Fig. 2A). This discrepancy is not explained by the capacity to form heterodimers with RXRs, as is the difference between the function of TRE1 and the ME TRE, both of which also have approximately equal affinities for TR binding (Fig. 2B and C and 3A). There is a similar discrepancy between the higher double occupancy by T3-TR on mutation 30 and the modest decrease in function relative to that of TRE1. These results demonstrate that the requirements for TRE1 function are more rigorous than those for the double occupancy of the half-sites by TR monomers in gel shift assays. This differs from results correlating homodimer binding with function that use more conventional TREs such as those that are found in the rat α myosin heavy chain, rGH, and ME enzyme promoters (13, 72). The explanation for this difference is not clear but could well relate to the subsequent interactions of closely spaced TR-TR homodimers or RXR-TR heterodimers with additional nuclear proteins to facilitate transcriptional events on more conventional TREs. These would occur less readily or not at all on the widely spaced monomeric binding sites of TRE1 and make transcriptional activation by such TREs more

sensitive to TRE mutations which cause minor changes in TR-DNA binding affinity.

TRE2 is a typical DR+4 TRE. TRE2 in *hdio1* is composed of two RGGTCA consensus hexamers conforming to a typical DR+4 arrangement. A DR+4 conformation TRE was first recognized in the rGH promoter by methylation interference footprinting by using partially purified rat hepatic T3 receptor (33). Subsequent studies illustrated that the 4-bp spacing dictated a preference for T3-TR activation by T3 as opposed to that by liganded RAR or vitamin D receptors (69). TR monomer binds preferentially to the 3′ half site of TRE2, which has the preferred YRRGGTCA consensus octamer sequence, in agreement with earlier studies of DR+4 TREs (2, 26, 70). Mutation of the T at −2 to A (mutation 10) has only modest effects on function (Fig. 7A) and none on heterodimer binding of RXRβ-TR complexes (Fig. 7B). However, this mutation does eliminate TR monomer binding on this element (Fig. 7B, lanes 4 to 6 versus lanes 1 to 3), illustrating the importance of this nucleotide for TR binding. This is consistent with the footprinting results (Fig. 7C) as well as earlier analyses of the functionally potent monomer TAAGGTCA (29) and of the T at −2 in a model DR+4 (26). Mutation of the core G nucleotides in the 5′ half-site of TRE2 (mutation 12) completely eliminates its function, indicating that a single TGAGGTCA half-site in this context is not sufficiently potent to act as a TRE despite its capacity to bind T3 TR (Fig. 7B). This suggests a critical role for A versus G at the −1 position of a monomeric element in conferring in vivo function but not in vitro binding (29). Mutation of the core G residues of the 3′ half-site (mutation 11) causes a significant loss of both homo- and heterodimer binding, as is the case for TRE1. However, for TRE2, it is not certain whether the absence of binding is due to decreased affinity for preformed soluble RXR-TR or TR-TR complexes or because monomer binding of TR must precede the binding of RXR or TR to the 5′ site (2, 19, 38, 73). The cooperativity for double occupancy of TRE2 is significantly higher than that of either TRE1 or ME (Fig. 2C). While TRE2 is more potent than TRE1 when placed immediately 5′ to the heterologous TK promoter, its position in the *hdio1* promoter some 700 nucleotides upstream of the TSS would be predicted to reduce its effectiveness. For example, we have previously shown a 60% reduction in the potency of a palindromic TRE when it is moved 700 bp 5′ to its original position upstream of the rGH promoter (12). Binding of cell-type-specific inhibitory nuclear proteins to sites intervening between TRE2 and the *hdio1* promoter might also interfere with its T3 responsiveness, and further studies will be required to address this issue.

While we and others have previously demonstrated that transient overexpression of TR can suppress basal transcription from TRE-containing homologous and heterologous promoters (10, 11, 17, 22), we did not observe this effect in the present studies using the TK promoter with TRE2 in JEG, HEK 293, or HepG2 cells. However, apo-TR-dependent repression does occur with the 716-nucleotide *dio1* promoter or when the TRE is placed 5′ to a minimal *dio1* promoter (data not shown). This difference is most likely due to the fact that TK is much stronger than is the TATA-less *dio1* promoter.

Physiological implications of the thyroid hormone responsiveness of the *hdio1* gene. The presence of two TREs close to the TSS implies an important physiological role for the T3 response of the *hdio1* gene. The 5′ monodeiodination of T4 to form the active thyroid hormone by D1 is required for thyroid hormone action, since T3 has a 10- to 15-fold higher affinity for TR than does T4 (61). However, it is not obvious why the product of this reaction should enhance a rate-limiting step in its own formation. Since D1 can also inactivate T4 or T3 by monodeiodination of the inner ring, lower D1 activity will prolong the half-life of T4 or T3 (48). When vertebrates become iodine deficient, compensatory alterations occur in the thyroid, resulting in the replacement of T4 synthesis by that of T3 (1, 58). While circulating T3 concentrations can remain remarkably constant via this process even when serum T4 is markedly reduced (58), with severe and prolonged deficiency, animals become modestly hypothyroid (50, 54). Accordingly, it is teleologically appropriate for D1 activity to decrease during iodine deficiency to prolong the half-life of whatever circulating T3 is formed in the thyroid and since monodeiodination of T4 by D1 in liver and kidney tissue is no longer an important pathway for T3 production. Since obtaining a sufficient supply of iodide for thyroid hormone synthesis has been (and continues to be) a major challenge for terrestrial vertebrates, it is possible that the T3 responsiveness of *dio1* evolved to mitigate its consequences. In agreement with this concept, a decrease in hepatic and renal D1 activity (as well as a thyrotropin-induced increase in the thyroidal D1) has been observed in iodine-deficient rats (54). Furthermore, the protective effects of a decrease in the selenoenzyme D1 in iodine deficiency have been illustrated by the adverse effects of selenium supplementation on thyroid function in individuals with combined nutritional deficiencies of selenium and iodine (16). Accordingly, the increase in D1 in patients with hyperthyroidism due to Graves' disease, which further increases T3 production and exacerbates thyrotoxic manifestations, may be viewed as a deleterious result of the fact that this autoimmune condition occurs exclusively in humans.

The presence of TRE1 in *hdio1* suggests that D1 will be T3 responsive even in tissues which do not express RXR-type proteins or in which these proteins are complexed to other nuclear receptors. Whether there are other nuclear proteins which could cobind with TR to TRE1 or similar response elements remains to be explored; however, these were not seen in JEG cells. Our results illustrate that the spacing between TR binding half-sites is only one of the factors which determines the capacity for a TRE-induced response to thyroid hormone. Thus, sequences containing consensus TR-binding octamers cannot be ignored as potential half-sites of a TRE merely because they do not conform to the classically spaced DR+4, palindromic, or everted palindromic configurations.

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