

# Identification of Direct Repeat 4 as a Positive Regulatory Element for the Human TR4 Orphan Receptor

A MODULATOR FOR THE THYROID HORMONE TARGET GENES\*

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**While the TR4 orphan receptor (TR4) is able to repress the expression of its target genes via its interaction with the direct repeat 1-hormone response element (DR1-HRE) and DR2-HRE, we now report that TR4 can also induce the transcriptional activity of the reporter gene containing a DR4-HRE via chloramphenicol acetyltransferase assay. Electrophoretic mobility shift assay and Scatchard analysis reveal a strong binding affinity (dissociation constant = 2 nM) between TR4 and DR4-HRE. The induction mediated by TR4 was detected not only in the synthetic DR4-HRE but also in some genes, such as rat  $\alpha$ -myosin heavy-chain and S14 genes, containing the DR4 or DR4-like motif, which have been suggested to be the response elements for a thyroid hormone receptor. Our data also demonstrate this TR4-mediated gene induction is TR4 dose- and DR4 sequence-dependent. Together, our data suggest that DR4-HRE can be a positive regulatory element for TR4, which may be able to induce the transcriptional activity of the genes containing such positive HREs.**

The nuclear receptor superfamily comprises a large group of ligand-dependent transcriptional factors that control the expression of target genes by binding to their cognate hormone response elements (HREs)<sup>1</sup> (1–4). It includes the receptors for steroid hormones, thyroid hormones, and retinoids. In addition to the classical receptors, a large number of members in this superfamily have been cloned via their sequence conservation, for which ligands were not found and therefore were classified as orphan receptors (5). The human TR4 orphan receptor (TR4) was initially isolated by our laboratory from human prostate and testis cDNA libraries by degenerative polymerase chain reaction cloning (6). TR4 shows a high degree of nucleotide

sequence homology with the TR2–11 orphan receptor and thus forms a unique subfamily in this superfamily (7). Recently, TR4 has also been identified from human lymphoblastoma cells, called TAK1 (8). Northern blot analysis shows that TR4 is expressed in many tissues, being predominantly located in granule cells of the hippocampus and the cerebellum (6). This implies that the TR4 may regulate the specific gene function in a cell-specific manner.

A cysteine-rich cluster that comprises two zinc finger-like structures can be easily identified within the DNA binding domain of the nuclear receptors (9–12). Three amino acids in the stem of the first zinc finger (the P box) are critical to specify the half-site sequence, while 5 amino acids in the second finger (the D box) can alter the selection pattern of the half-site spacing. On the basis of its distinct P box, TR4 can be classified into the estrogen receptor/thyroid hormone receptor subfamily which displays cross-recognition of the palindromic or direct repeat (DR) HREs. These HREs consist of a core half-site, AGGTCA, or related sequence (13–15). Our previous data suggested that TR4 preferred to bind to the HREs, which consist of two DRs of consensus half-site, rather than a palindrome.<sup>2</sup> Furthermore, TR4 can repress the retinoic acid-induced transactivation by competitive DNA binding to the response elements of other receptors (*i.e.* DR5 for retinoic acid receptor (RAR) and DR1 for retinoid X receptor (RXR)).<sup>2</sup> In addition, the TR4-mediated suppression can also be found in the gene promoter containing DR2-like response element that is located in the +55 region of the SV40 major late promoter (16).

All of the above findings suggest that TR4 may function as a repressor to block other receptor-mediated transcriptional activities through competitive DNA binding to the same HREs. In the present study, we tested the regulation of TR4 on a DR4-HRE which has been shown to be a hormone response element for thyroid hormone receptor (T<sub>3</sub>RE) by using a reporter gene assay (17–19). Through these studies, we found that the TR4 can induce gene expression through the interaction with DR4-HRE in a sequence- and dose-dependent manner. In addition to repressing its target genes, our data provide the first evidence to show that TR4 can also activate its target genes, through a DR4- or DR4-like T<sub>3</sub>RE. This finding may expand our understanding of the physiological function of the TR4 orphan receptor.

## MATERIALS AND METHODS

**Plasmid Construction**—For the transient transfection or coupled *in vitro* transcription and translation of the full-length TR4 protein, the pCMX-TR4 and pET14b-TR4 plasmids were constructed as described, respectively (16). pCD-TR $\alpha$ 1, the expression vector for thyroid hormone

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<sup>1</sup> The abbreviations used are: HRE, hormone response element; TR4, TR4 orphan receptor; DR, direct repeat;  $\alpha$ -MHC,  $\alpha$ -myosin heavy chain; FUR, far upstream regulatory region; HIV, long terminal repeat of the human immunodeficiency virus I; T<sub>3</sub>R, thyroid hormone receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; RA, retinoic acid; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase.

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receptor  $\alpha$ , was provided by Dr. Leslie J. Degroot. The reporter plasmids -32TK-CAT and TK-(DR4)<sub>2</sub>-CAT, either without or with two copies of DR4 (5'-GATATAGGGGTCAAATAAGGTCAAATG-3', Fig. 1A), were kindly provided by Dr. RC. Ralff. TK-(mDR4)-CAT containing one copy of the mutated DR4 (5'-GATCTAGGGCTCAAATAAGGTCAAATG-3') inserted into the *Bam*HI site of -32TK-CAT plasmid was generated as a negative control. The reporter genes used pCATp reporter genes fused with either the 5'-flanking region of rat  $\alpha$ -myosin heavy chain ( $\alpha$ -MHC) (from -374 to +420) (17), the far upstream regulatory region (FUR) (from -2952 to -2448) in the rat S14 gene (FUR1/2/3-CAT) (18), or the long terminal repeat of the human immunodeficiency virus I (from -454 to +82, HIVLAILTR.CAT) (19), which contains the T<sub>3</sub>RE motif in the natural promoter region.

**Coupled *In Vitro* Transcription and Translation**—Expression plasmids pET14b-TR4 and pCD-TR4 $\alpha$ 1 were *in vitro* transcribed and translated by the TNT system (Promega) as described previously (16).

**Electrophoretic Mobility Shift Assay (EMSA)**—EMSA analysis was performed according to the methods described by Cooney *et al.* (20). In general, the double-stranded DR4 oligonucleotides were end-labeled with a [ $\gamma$ -<sup>32</sup>P]ATP (specific activity = 6000 Ci/mmol, DuPont NEN) by T4 polynucleotide kinase to 2–8 × 10<sup>8</sup> cpm/ $\mu$ g. For competitive DNA binding reactions, 100-fold excess of cold double-stranded DR4 or mDR4 oligonucleotides were mixed with the proteins prior to the addition of labeling probe to the reactions. For antibody supershift analysis, 1  $\mu$ l of the monoclonal anti-TR4 antibody (G232–303.4) was added to the reaction. As a control for supershift assays, the monoclonal anti-androgen receptor receptor antibody (N1–15) (21) was added. Gels were fixed in 50% ethanol and 10% acetic acid for 30 min before drying. The radioactive gels were analyzed by either PhosphorImager (Molecular Dynamics Inc.) or autoradiography.

**Scatchard Analysis**—The DNA-protein binding assay was performed as described previously (16). In brief, free probes (<sup>32</sup>P-DR4) and DNA-protein complexes were resolved in native gel and quantified by scintillation counting. The dissociation constant ( $K_d$ ) was determined from the minus reciprocal of the slope of the line generated from the empirical data.

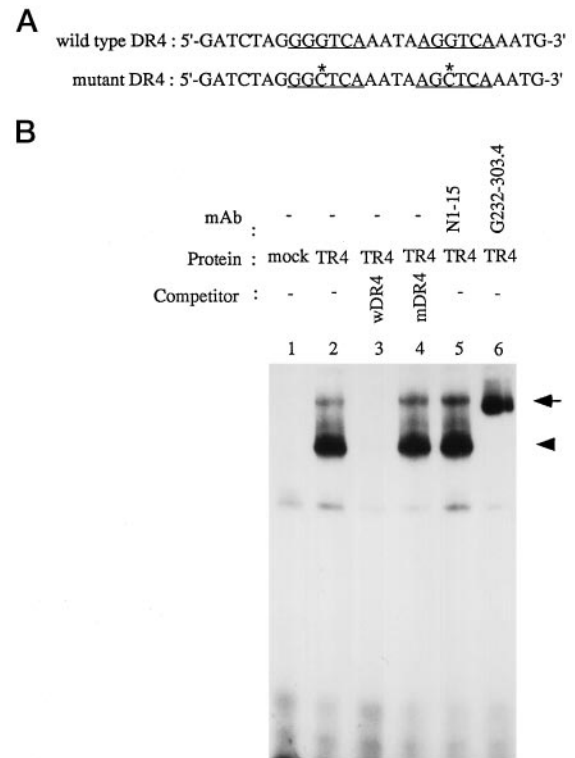
**Transient Transfection**—HepG2 cells were routinely maintained in Dulbecco's modified Eagle's medium with 5% heat-inactivated fetal bovine serum. HepG2 cells (3 × 10<sup>5</sup>) were seeded in 6-cm culture dishes 24 h before transfection. The medium was changed to Dulbecco's modified Eagle's medium with 5% charcoal dextran-treated serum at least 1 h before transfection. The cells were transfected using a modified calcium phosphate precipitation method (22). To normalize the transfection efficiency, the  $\beta$ -galactosidase expression vector was co-transfected.

**Statistics**—The data are presented as mean  $\pm$  S.D. One-way analysis of variance was used to determine the differences among multiple groups. Student's *t* test was used for analyzing differences between two groups. The degree of significance (*p* value) was shown with asterisks. *p* < 0.05 was accepted as the level of statistical significance.

## RESULTS

**TR4 Specifically Binds to DR4, the AGGTCA Motif with 4-Nucleotide Spacing**—EMSA was used to determine binding specificity for TR4 to DR4-HRE. *In vitro* translated TR4 protein was incubated with <sup>32</sup>P-labeled DR4-HRE and analyzed on a 5% polyacrylamide gel. As shown in Fig. 1B, a specific DNA-protein complex (indicated with arrowhead, lane 2) was identified that has a migration distinct from the nonspecific DNA-protein complexes in the presence of mock-translated product (lane 1). The binding specificity was further confirmed by adding excess unlabeled DR4-HRE or mutated DR4 (mutated DR4-HRE, in which the third G was replaced with C) (Fig. 1A). Competition by 100-fold excess unlabeled DR4-HRE abolished the specific DNA protein complex (lane 3). In contrast, this complex could not be abolished completely with the unlabeled mutant DR4-HRE (lane 4). This DNA-protein complex could not be affected when androgen receptor monoclonal antibody (N1–15) was added (lane 5). In addition, when the anti-TR4 monoclonal antibody (G232–303.4) was added in the reaction, a supershifted band made by DNA-receptor-antibody complex was visible (lane 6).

All these data clearly demonstrated that the TR4 can bind specifically to DR4-HRE. We also calculated the binding affin-



**FIG. 1. Binding of *in vitro* expressed TR4 to DR4.** A, the sequences of the wild type DR4 and mutated DR4 oligonucleotides. B, <sup>32</sup>P-labeled DR4 oligomers were used in EMSA with 1  $\mu$ l of *in vitro* translated TR4. A 100-fold excess unlabeled wild type DR4 (*wDR4*) (lane 3) or mutant DR4 (*mDR4*) (lane 4) was added as a competitor. 1  $\mu$ l of anti-TR4 monoclonal antibody (G232–303.4) was also added (lane 6) and anti-androgen receptor monoclonal antibody (N1–15) was used as a control (lane 5). The migration position of the specific binding formed by the DNA-protein complex and the supershifted band formed by adding monoclonal antibody are indicated as an arrowhead or arrow, respectively.

ity between TR4 and DR4-HRE by the Scatchard analysis. Fixed amounts of *in vitro* translated TR4 proteins were incubated with an increasing amount of <sup>32</sup>P-labeled DR4 (0.1–12.8 ng) and resolved in EMSA. As shown in Fig. 2, Scatchard analysis displays a single binding component with a dissociation constant ( $K_d$ ) of 2 nM and  $B_{max}$  of 0.075 nM. These data fit the  $K_d$  range for steroid receptors and their HREs.

**TR4 Mediates Dose-dependent TK-(DR4)<sub>2</sub>-CAT Transcriptional Activation**—As shown above, TR4 can bind to a DR4-HRE with high specificity and affinity. We were then interested in knowing the consequences of this binding. The reporter gene TK-(DR4)<sub>2</sub>-CAT and the TR4 expression (pCMX-TR4) plasmid were co-transfected into HepG2 cells. Surprisingly, we found that TR4 itself was able to activate CAT activity (Fig. 3A, lane 3). This transactivation could not be observed when pCMX-TR4 was co-transfected with the reporter plasmids without DR4-HRE (-32TK-CAT, lane 1), or with the mutated DR4-HRE (TK-(mDR4)-CAT, lane 2). Moreover, the CAT activity was increased as increasing amounts (from 0.01 to 1  $\mu$ g) of pCMX-TR4 were co-transfected (Fig. 3B, lanes 1–6). Transcriptional activity reached a plateau when 2  $\mu$ g of pCMX-TR4 was co-transfected (lane 7). In summary, these data clearly demonstrated that TR4 induced transcriptional activation is TR4 dose- and DR4-HRE sequence-dependent.

**The Correlation between TR4 and TR4 $\alpha$ 1 in the Transactivation of TK-(DR4)<sub>2</sub>-CAT**—All of the data above suggest that DR4-HRE is a positive novel response element for the TR4. Previous reports suggest that the DR4-HRE can also serve as the response element for the T<sub>3</sub>RE. Therefore we investigated

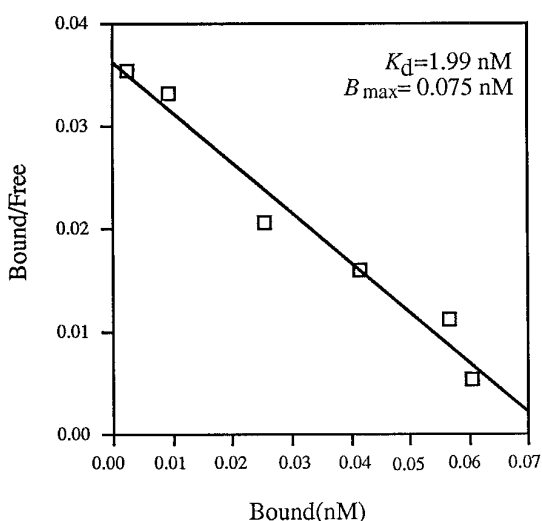


FIG. 2. **Scatchard analysis of TR4 to DR4.** 1  $\mu$ l of *in vitro* translated TR4 protein was incubated with serially diluted  $^{32}$ P-labeled DR4 (concentrations from 0.1 to 12.8 ng), and resolved in EMSA. After autoradiography, the respective bands were excised, placed in scintillation fluid, and quantified directly in a scintillation counter. The ratio activity between specific DNA-protein binding (*bound*, nM) and free DNA probe with respect to specific DNA-protein binding (*bound/free*) was plotted. The dissociation constant ( $K_d$ ) and  $B_{max}$  values were generated from the Edba program (Biosoft).

how these two receptors interacted with each other on the same target sequence by designing the following experiments. First, a fixed amount (2  $\mu$ g) of thyroid hormone receptor expression vector, pCD-TR $\alpha$ 1, was transfected singly or co-transfected with increasing amounts of pCMX-TR4 (from 0.125 to 1  $\mu$ g) in the absence of T<sub>3</sub>. As shown in Fig. 4A, CAT activities were activated only when pCMX-TR4 was transfected (*lane 1 versus 2*). No activation was detected when pCD-TR $\alpha$ 1 was transfected alone (*lane 1 versus 3*). The induction was significant increasing ( $p < 0.005$ ) by increasing amounts of pCMX-TR4 (*lanes 3–7*). The induced CAT activities mediated by TR4 were slightly reduced when the unliganded pCD-TR $\alpha$ 1 (2  $\mu$ g) was co-transfected (*lane 2 versus 7*). However, the reduction of TR4-mediated CAT activation by unliganded TR $\alpha$ 1 was not very significant ( $p < 0.1$ ) by Student's *t* test. This phenomenon was also observed by co-transfection of pCMX-TR4 (1  $\mu$ g) with increasing amounts of unliganded pCD-TR $\alpha$ 1 (Fig. 4C, *lanes 3–7*). The activities induced by TR4 (1  $\mu$ g) was reduced 50% when 2.0  $\mu$ g of unliganded TR $\alpha$ 1 was co-transfected. (Fig. 4C, *lane 3 versus 7*). Again, the dose of unliganded TR $\alpha$ 1 does not significantly ( $p < 0.1$ ) repress the activation of CAT activities by TR4. This may be due to the fact that TR4 is more potent than liganded TR $\alpha$ 1 in the activation of TK-(DR4)<sub>2</sub>-CAT reporter gene. The tendency for repression of TR4-mediated transcriptional activity by the unliganded TR $\alpha$ 1 is consistent with previous reports (23–25) in which suggested that unliganded TR $\alpha$ 1 may act as a gene silencer.

In contrast, in the presence of T<sub>3</sub> (10<sup>-7</sup> M), both pCD-TR $\alpha$ 1 and pCMX-TR4 can activate CAT activities up to 15- and 22-fold alone, respectively (Fig. 4, B and D, *lanes 1–3*). The transcriptional activity slightly increased when we co-transfected 2  $\mu$ g of pCD-TR $\alpha$ 1 and increasing amounts of pCMX-TR4 (0.125 to 1  $\mu$ g) (Fig. 4B, *lanes 3–7*). Furthermore, when a fixed amount of pCMX-TR4 (1  $\mu$ g) was transfected together with increasing amounts of pCD-TR $\alpha$ 1 (from 0.5 to 2  $\mu$ g) (Fig. 4D, *lanes 3–7*), the results showed that CAT activity induced by TR4 did not increase significantly by adding additional TR $\alpha$ 1. This may suggest that transcriptional activity could be saturated by expression of 1  $\mu$ g of TR4 alone.

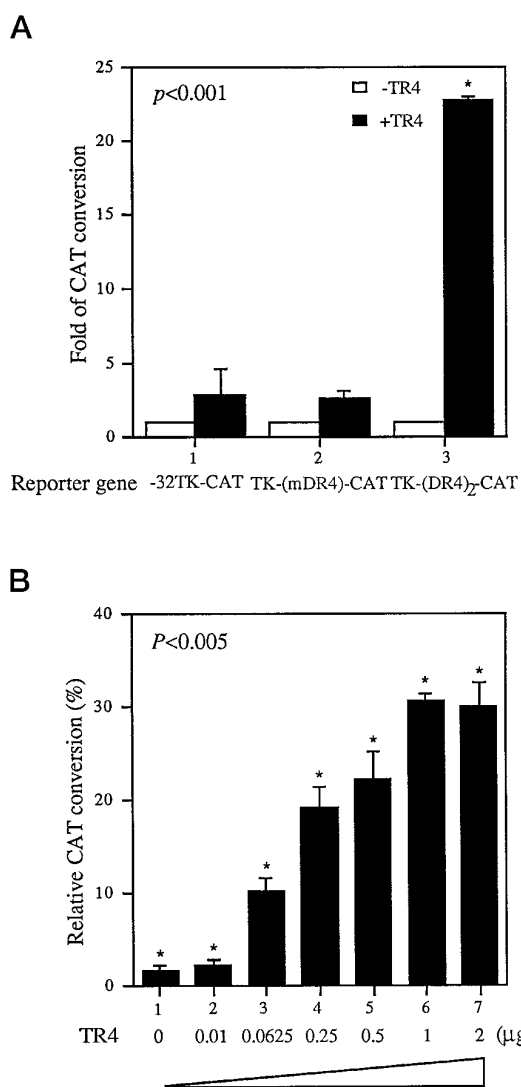
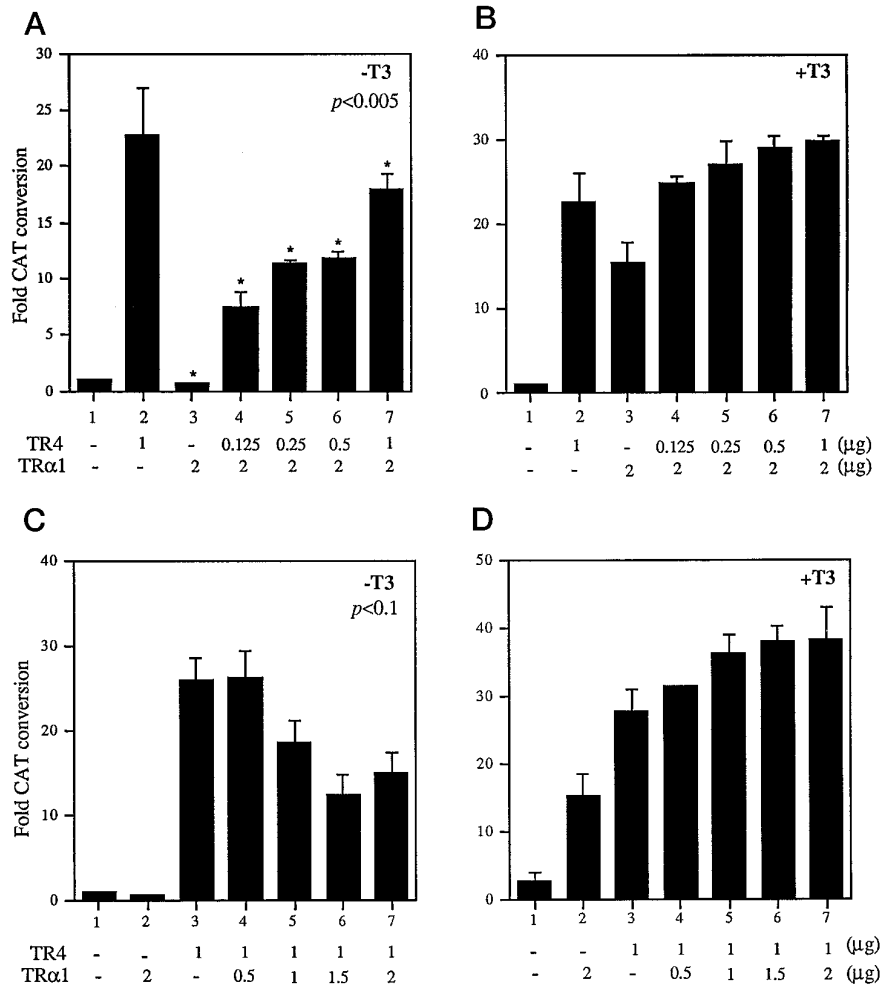


FIG. 3. **T<sub>3</sub>-independent activation of TK-(DR4)<sub>2</sub>-CAT by TR4.** A, -32TK-CAT, TK-(mDR4)-CAT, or TK-(DR4)<sub>2</sub>-CAT reporter plasmids (3  $\mu$ g of each) were transfected into HepG2 cells with or without co-transfection of TR4 expression vector (pCMX-TR4, 1  $\mu$ g). At the 3rd day after transfection, cells were harvested for determination of CAT activities. The relative CAT conversion was determined by comparing the CAT activity in each treatment with the one without co-transfection of 1  $\mu$ g of pCMX-TR4. Significant differences were determined by Student's *t* test and marked an asterisk. \* $p < 0.001$  compared with pSG5-transfected groups. B, dose response of TK-(DR4)<sub>2</sub>-CAT activation by TR4. HepG2 cells were transfected with TK-(DR4)<sub>2</sub>-CAT reporter plasmids (3  $\mu$ g of each) and co-transfected with indicated increasing amounts of pCMX-TR4 (from 0 to 2  $\mu$ g). The percentage of CAT conversion was plotted. All CAT assays were normalized for the level of  $\beta$ -galactosidase activity. Significant differences ( $p < 0.005$ ) of CAT activity induced by TR4 at different doses were determined by one-way analysis of variance. Each value represents the mean  $\pm$  S.D. of four independent experiments.

**TR4 Activates the Transcriptional Activities of Some Genes Containing T<sub>3</sub>RE Motif**—As described above, TR4 can recognize synthetic DR4-HRE and activate TK-(DR4)<sub>2</sub>-CAT reporter gene in a dose- and DNA sequence-dependent manners. We then explored whether this TR4-mediated CAT induction could also be applied to the DR4-T<sub>3</sub>RE-containing or nonclassical T<sub>3</sub>RE natural promoters. The sequences corresponding to T<sub>3</sub>RE were shown in Fig. 5A with an arrow or underline. The first gene used was from the 5'-flanking region of rat  $\alpha$ -MHC gene (from -347 to +420), which contains a DR4-HRE that serves as a T<sub>3</sub>RE (17). The expected results were shown in Fig. 5B, panel a. The transcriptional activity of this gene can be induced up to

**FIG. 4. The combination effect of TR4 and thyroid hormone receptor (TR $\alpha$ 1) on TK-(DR4)<sub>2</sub>-CAT.** TK-(DR4)<sub>2</sub>-CAT (3  $\mu$ g) was co-transfected into HepG2 cells with pSG5, pCMX-TR4, or pCD-TR $\alpha$ 1. In panels A and B, fixed amounts of TR $\alpha$ 1 (2  $\mu$ g) were co-transfected with indicated increasing amounts of pCMX-TR4 (from 0 to 1  $\mu$ g). In panels C and D, fixed amounts of pCMX-TR4 (1  $\mu$ g) and increasing amounts of pCD-TR $\alpha$ 1 (from 0 to 2  $\mu$ g) were co-transfected as indicated. Twenty-four hours after transfection, the cells were left untreated (panels A and C) or treated (panels B and D) with  $10^{-7}$  M T<sub>3</sub> and harvested for determination of CAT activities. All CAT assays were normalized for the level of  $\beta$ -galactosidase activity. In panel A, the dose of TR4 significantly ( $*p < 0.005$ ) affects the induction of DR4-CAT reporter gene activity in the presence of unliganded TR $\alpha$ 1. In panel C, the dose of unliganded TR $\alpha$ 1 does not significantly ( $p < 0.1$ ) repress the induction of DR4-CAT activity by 1  $\mu$ g of TR4. Each value represents the mean  $\pm$  S.D. of three independent experiments. The induction fold was compared with the transfection of TK-(DR4)<sub>2</sub>-CAT plus pSG5. Significant differences of CAT activity affected by TR4 or TR $\alpha$ 1 at different doses were determined by one-way analysis of variance.



9.5-fold with co-transfection of pCMX-TR4. The second gene tested was the FUR of the rat S14 gene (from -2952 to -2448) which contains multiple T<sub>3</sub>REs that synergize with each other in their responsiveness to T<sub>3</sub> (18, 26). As shown in Fig. 5B, panel b, the transcriptional activity of rS14 can be induced up to 6.3-fold upon co-transfection with pCMX-TR4. The third native gene we tested was the HIV-LTR (from -454 to +82), which contains a nonclassical T<sub>3</sub>RE, GGCGGG, and can be activated by unliganded TR $\alpha$ 1 (27). A 3.9-fold induction mediated by pCMX-TR4 can also be observed (Fig. 5B, panel c). The differences of CAT activity obtained between TR4-transfected and control in these three native T<sub>3</sub>RE responsive genes were considered significant ( $p < 0.05$ ) based by Student's *t* test. All these data suggest that the DR4-T<sub>3</sub>RE as well as some nonclassical T<sub>3</sub>REs can serve as potential target sequences for both T<sub>3</sub>R and TR4.

To further define the correlation between TR4 and TR $\alpha$ 1 in these natural promoter constructions, we then tested the overexpression of both pCMX-TR4 and pCD-TR $\alpha$ 1 in the reporter gene assay. Fig. 6A showed that CAT activities were significantly ( $p < 0.05$ ) induced by the transfection of pCMX-TR4 alone in all reporter plasmids we had tested, but induction could not be observed in the transfection of unliganded pCD-TR $\alpha$ 1. In the absence of T<sub>3</sub>, the activation induced by TR4 was slightly reduced by unliganded TR $\alpha$ 1 in synthetic DR4,  $\alpha$ -MHC, and S14 reporter gene constructs but not in HIV-LTR reporter gene. These results agree with a previous report in which HIV-LTR was shown to be activated by unliganded TR $\alpha$ 1, and the addition of T<sub>3</sub> could reverse this effect (27). On the other hand, in the presence of T<sub>3</sub> (Fig. 6B), cotransfection of

pCMX-TR4 with pCD-TR $\alpha$ 1 slightly increased the activation as compared with the single receptor transfection but the increasing effect is not very significant. Together, our data provides the first evidence showing that the TR4 is capable of activation of CAT reporter genes containing a DR4-T<sub>3</sub>RE. This is true for both synthetic and native T<sub>3</sub>REs as well as other nonclassical T<sub>3</sub>REs.

#### DISCUSSION

Like the TR2 orphan receptor, TR4 can also serve as a repressor in RAR/RXR-mediated gene induction and transcription from the major late promoter of the SV40+55 gene (16, 22, 28). In the present study, we showed TR4 can also induced the transcriptional activity of genes containing a DR4 or DR4-like sequence. These data suggest that the selectivity of the receptor response is dependent on the core motif, including the spacing, orientation, and precise sequence composition of the adjacent core motifs. In Fig. 1, when we replaced the third G with C in DR4-HRE (mDR4), an excessive amount of unlabeled mDR4 could not abolish with the specific DNA-receptor complex completely. This suggests that the precise sequence of the core motif plays an essential role in TR4-mediated gene regulation, a result in agreement with our previous studies as the TR2 orphan receptor (22, 28). In addition, the spacing of core binding motifs may also be a key factor in dictating specificity of transcriptional response to a transcriptional factor. TR4 can either induce or suppress transcription through the binding to HREs with same sequence of the core binding motif but different spacing. Activation or repression may be dependent on different spacing of the motifs (DR1/DR5 for the repression of



heterodimers. In contrast, RXR is active as a non-DNA-binding co-factor with the NGFI-B/nurr1 orphan receptors (35). Although we were unable to demonstrate the interaction between RXR and TR4, it is still possible that TR4 may need other co-factors for proper function. Using a yeast-two hybrid system, we are in the process of isolating several TR4-associated proteins.<sup>3</sup> Recently, COUP-TF has been shown to inhibit the transcriptional activities of T<sub>3</sub>R, 1,25-dihydroxyvitamin D<sub>3</sub> receptor (36), RAR, RXR (37), the peroxisome proliferator-activated receptor (38, 39), and hepatocyte nuclear factor 4 (40). The molecular mechanism of COUP-TF-mediated transcriptional repression may also be involved in some other activator(s) (41). Together, these findings suggest that interactions among proteins may contribute to receptor-mediated gene induction or suppression.

Ligands have always played a central role in the activation of steroid hormone receptors. For example, unliganded T<sub>3</sub>R is associated with cellular co-repressor p270/N-CoR which serves as a gene silencer (24). Upon ligand binding, T<sub>3</sub>R dissociates with the co-repressor and actively turns on its target genes (23, 42). However, a similar effect was also observed in this study. Unliganded TR $\alpha$ 1 has a potential suppressive effect on the TR4-mediated transactivation in both synthetic DR4 or native T<sub>3</sub>RE-CAT reporter genes, although the effect is not very significant ( $p < 0.1$ ). One of the potential explanation is that TR4 has higher binding affinity than TR $\alpha$ 1 to T<sub>3</sub>REs. Therefore, TR4 may compete stronger than TR $\alpha$ 1 on binding to the same HRE to activate its target gene. On the other hand, the physiological function of orphan receptors still remains obscure because information regarding ligand is lacking. Nevertheless, the induced CAT activity shown in this study may still provide us a useful assay system for searching for potential ligands or activators for the TR4 orphan receptor.

What then is the relationship between TR4 and TR $\alpha$ 1, since they both recognize the same HRE? Transient transfection studies show that TR4 can activate CAT activity in the presence or absence of T<sub>3</sub> suggesting that T<sub>3</sub> is not the ligand or activator for TR4. In contrast, T<sub>3</sub>R can only activate transcriptional activity when the ligand, T<sub>3</sub>, is present. These results suggest that two different receptors may use different ligands to induce similar or different sets of target genes. Another possibility is that although two different receptors can recognize the same HRE, they may function differently and independently in gene regulation. For example, T<sub>3</sub>R can also bind with high affinity to estrogen response element and serves to antagonize estrogen-dependent transcriptional activation (15). In our present study, we found that the T<sub>3</sub>-induced transactivation of TR $\alpha$ 1 could be overwhelmed by TR4. In addition, the repression effects from unliganded TR $\alpha$ 1 could be overcome by adding more TR4. The dominant effect of TR4 may be due to its higher binding affinity to DR4-HRE as compared with that of TR $\alpha$ 1 (data not shown). Together, our data suggested TR4 may play an important role in TR $\alpha$ -mediated gene regulation.

In summary, our data suggest that the ability of TR4 to induce genes with T<sub>3</sub>RE-DR4-HRE may provide us with a model system for future studies in determining the mechanisms to determine how the TR4 activates and represses its target genes. Moreover, this TR4-mediated induction system may provide a potential assay for finding a possible ligand and/or activators for TR4.

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