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TR4 Orphan Receptor Crosstalks to Chicken Ovalbumin Upstream Protein-Transcription Factor and Thyroid Hormone Receptor to Induce the Transcriptional Activity of the Human Immunodeficiency Virus Type 1 Long-Terminal Repeat

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Here we investigate the roles of human testicular orphan receptors, TR2 and TR4, on the gene regulation of the long-terminal repeat of the human immunodeficiency virus type 1 (HIV-LTR). In gel-retardation assays, a palindromic element at the 5'-end of HIV-LTR, 5'-AGGGGTCAGATATCCACTGACCTTT-3', showed high affinity to TR2 and TR4 with an equilibrium dissociation constant (K_d) of 1.11 ± 0.48 (n = 3) and 0.52 ± 0.12 n*M* (n = 3), respectively. Interestingly, each half-site of the palindromic element is sufficient to compete with the binding of the labeled palindromic element to TR2 or TR4 with an equilibrium inhibition constant (k_i) around 10 n*M*. However, the transiently expressed TR2 or TR4 in Chinese hamster ovary (CHO) cells or Japanese quail muscle myoblasts (QM7) cells showed no activity in regulating the transcriptional activity of the chloramphenicol acetyltransferase (CAT) reporter gene inserted downstream of the HIV-LTR promoter. Although both TR2 and TR4 showed no effect on CAT activity by itself, our data showed only the TR4 could crosstalk to the chicken ovalbumin upstream protein-transcription factor (COUP-TF1) and thyroid hormone receptor (TRα1), and potentiated the transcriptional activity of HIV-LTR on the CAT reporter gene regulated by COUP-TF1 and TRα1. These results indicate that TR4, but not TR2, may couple to other nuclear receptors in the upregulation of the HIV replication.

Key Words: TR2; TR4; HIV-LTR; COUP-TF1; TRα1.

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Introduction

The steroid-thyroid hormone receptor superfamily, including androgen receptor, estrogen receptor, glucocorticoid receptor, progesterone receptor, mineralocorticoid receptor, thyroid receptor, vitamin D receptor, and retinoid acid receptor, plays important roles in the control of vertebrate cell differentiation and tissue development *(1)*. These receptors act as *trans*-regulators of the transcription. On binding with their cognate ligands, the biological actions of these receptor complexes are mediated through the interaction with *cis*-acting DNA elements, called hormone response elements (HREs), to modulate the transcription of target genes. The functional structure and organization of these receptors comprise a variable N-terminal domain involved in the modulation of gene expression, a well-conserved DNA binding domain with two zinc fingers, and a partially conserved C-terminal ligand binding domain *(2)*.

Human testicular orphan receptors, TR2 and TR4, were isolated from human prostate and testis cDNA libraries with a probe designed to select clones encoding the steroid receptor DNA binding domain *(3,4)*. Both human TR2 and TR4 receptors have a similar size (67 kDa in molecular weight) and contain the characteristic domain features of steroid hormone receptors. Since no ligands have been identified, TR2 and TR4 belong to the orphan receptor family. The amino acid sequence analysis shows high homology between TR2 and TR4 orphan receptors, but different amino termini with no similarity with the other class of receptors, suggesting that these two orphan receptors constitute a unique subfamily within the steroid hormone receptor superfamily.

The TR2 orphan receptor has been demonstrated to be expressed in many rat tissues with higher abundance in male reproductive organs *(3)*. Unlike TR2 orphan receptor, TR4 is more highly expressed in the granule cells of the hippocampus and the cerebellum than in the male reproductive organs *(4)*. In embryos, both TR2 and TR4 have

similar overall expression patterns, but different kinetics. Within the central nervous system, TR2 is expressed mainly in the early embryonic stage, whereas TR4 is expressed in the postnatal stage *(5,6)*. In addition, both TR2 and TR4 are highly expressed in the proliferation-active population of most developing organs, whereas the expression is dramatically reduced in the more differentiated cell types *(5,6)*. In adult testis, both TR2 and TR4 are expressed predominantly in a population undergoing active meiosis. Also, in an in vitro assay, the TR2 promoter activity is rapidly repressed by radiation *(7)* and p53 may be responsible for such regulation.

Infection with human immunodeficiency virus type 1 (HIV-1) leads slowly, but inexorably to the loss of immune competence whose most striking feature is the loss of CD4 T-cells, and finally resulting in deterioration of patients immune and neurological status *(8)*. Once the virus is in the cell, reverse transcriptase synthesizes a double-stranded DNA version of its RNA genome. It is this provirus that integrates into the host cell DNA and becomes a permanent part of the host cell's genome. This provirus is presumably replicated along with the host DNA every time the cell divides. Although some viral replication occurs in lymphoid tissue, it could also replicate elsewhere, for example, in the brain, and in the male and female reproductive tracts. TR2 and TR4 are highly expressed in the brain and the human reproductive organs. These are the two organs shown to be highly active in HIV-1 replication. Both TR2 and TR4 have also been demonstrated to regulate the expression of simian virus 40 (SV40) major late promoter *(9,10)* as well as the transcriptional activity of human papillomavirus type-16 (HPV-16) long control region *(11)*. In addition, there exists a palindromic element at the 5'-end of HIV-LTR, 5'-AGGGGTCAGATATCCACTGACCTTT-3'. Chicken ovalbumin upstream protein-transcription factor (COUP-TF1) orphan receptor has been shown to regulate the transcriptional activity of HIV-LTR through the binding to this palindromic element in HIV-LTR *(12–14)*. Based on the above data, we speculate TR2 and TR4 may, like COUP-TF orphan receptor, also play some roles in the regulation of HIV-LTR. Our data indicate that neither TR2 nor TR4 alone showed any induced activity on the HIV-LTR promoter activity, although both can bind with high affinity to a palindromic element in the HIV-LTR promoter region. It is interesting to find that TR4, but not TR2, synergistically potentiates the transcriptional activity of HIV-LTR with COUP-TF1 and TRα1.

Results

TR2 and TR4 Bind Specifically to the Palindromic Element Oligonucleotide of HIV-LTR with High Affinity

Gel retardation assays were performed with the in vitro translated TR2 and TR4 using ^{32}P-labeled palindromic element (HIV-5,6) as the probe (Table 1). Figure 1A shows

Table 1
Sequences of Synthetic Oligonucleotides Used in this Study

Name	Nucleotide sequences
DR1	5'-AGCTTCAGGTCAGAGGTCAGAGAGCT-3'
HIV-3,4	5'-GATCAGGGCCAGGGGTCAGATATCCA-3'
HIV-5,6	5'-AGGGGTCAGATATCCACTGACCTTT-3'
HIV-7,8	5'-GATATCCACTGACCTTTGGATGGT-3'

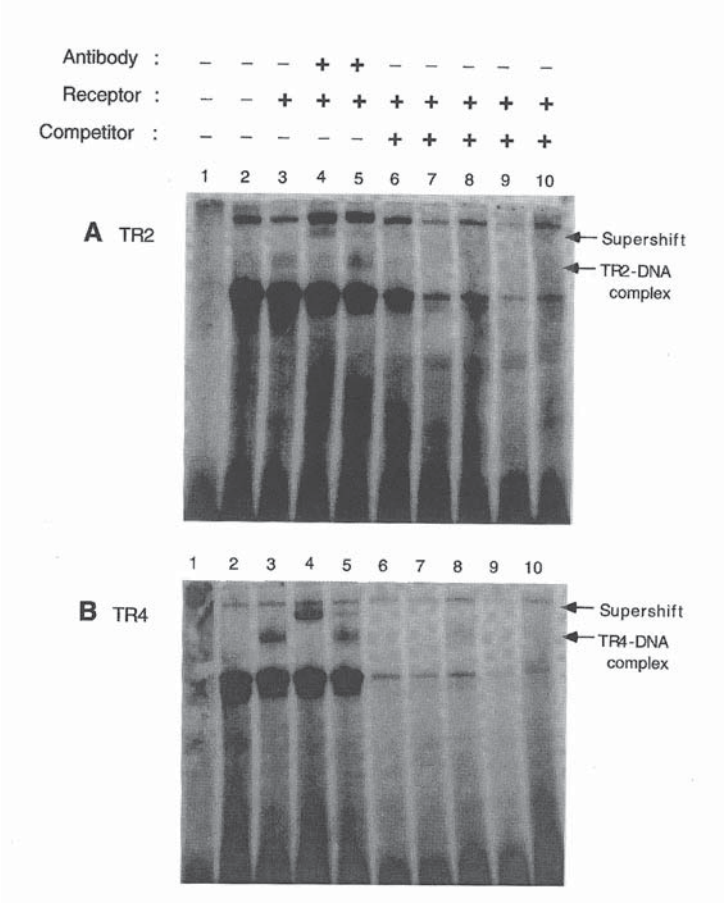

Fig. 1. Binding of the in vitro translated TR2 **(A)** and TR4 **(B)** to the ^{32}P-labeled palindromic element (HIV-5,6). For the binding studies, 0.1 ng of the ^{32}P end-labeled palindromic element and 1 μL of the in vitro translated TR2 (A) or TR4 (B) were incubated at room temperature for 30 min before loading to the 5% native polyacrylamide gel to separate bound and unbound ^{32}P-labeled oligonucleotides. For antibody supershift analysis, the antiserum from rabbit immunized with TR2 or MAb #29 anti-TR4 antibody was preincubated with the in vitro translation products at room temperature for 15 min before the addition of the probe. An unimmunized rabbit serum was used as the control in the TR2 supershifting studies. In the TR4 supershift experiment, an androgen receptor MAb, NI 15, was used as the control. Lane 1: probe alone; lane 2: labeled probe and the mock translated control; lane 3: probe and in vitro receptor translated product; lane 4: receptor, the probe, and the receptor specific antibody; lane 5: receptor, probe, and control antibody; lane 6: receptor, probe, and a 10-fold molar excess of HIV-5,6; lane 7: receptor, probe, and 100-fold molar excess of HIV-5,6; lane 8: receptor, probe, and 10-fold molar excess of HIV-7,8; lane 9: receptor, probe, and 100-fold molar excess of HIV-7,8; lane 10: receptor, probe, and 100-fold molar excess of HIV-3,4.

that a specific TR2-DNA complex was present in the presence of TR2 (lane 3), but was absent in the mock translated control (lane 2). This TR2 and DNA complex was no longer visible with either 10- or 100-fold molar excess of the unlabeled HIV-5,6 (lanes 6 and 7, respectively). Interestingly, the labeled-DNA binding to TR2 could also be specifically inhibited by either half-site of the palindromic element at 10- or 100-fold molar excess of HIV-7,8 (lane 8 and lane 9, respectively) or at 100-fold molar excess of HIV-3,4 (lane 10). Moreover, this retarded complex could be fully supershifted in the presence of the rabbit TR2 antiserum (lane 4), but could not be observed with the preimmuned rabbit serum (lane 5).

Figure 1B shows a similar binding characteristic of TR4 to the ^{32}P-labeled HIV-5,6. A specific TR4-DNA complex was observed in the presence of TR4 and the probe (lane 3 in Fig. 1B), which was not observed in the reaction of the probe with the mock-translated control (lane 4 in Fig. 1B). In the presence of the anti-TR4 monoclonal antibody (TR4 MAb #29), the TR4-DNA probe complex was supershifted to the upper part of the gel (lane 4). For an unknown reason, a minor supershift could also be observed with an androgen receptor specific MAb (NI15) in this experiment (lane 5). Again, similar to the case of TR2, the TR4-DNA complex could be fully abolished with either 10- or 100-fold excesses of the unlabeled HIV-5,6 (lane 6 and lane 7, respectively) as well as the unlabeled oligonucleotides containing 10- to 100-fold molar excess of the downstream half-site (HIV-7,8) (lane 8 and lane 9, respectively) and 100-fold molar excess of the upstream of the half-site (HIV-3,4) (lane 10) of the palindromic element. A 10-fold molar excess of HIV-7,8 is apparently not enough to eliminate the TR4 binding to the palindromic element (HIV-5,6). The TR4-DNA complex remained visible in lane 8.

Figure 2 shows Scatchard plots of the specific binding of the ^{32}P-labeled HIV-5,6 oligonucleotide to TR2 (Fig. 2A) and TR4 (Fig. 2B). Linear Scatchard plots were obtained for both TR2 and TR4, indicating the presence of a single population of binding sites. The equilibrium dissociation constants (K_d) as calculated from the inverse of the slope for TR2 and TR4 were 1.11 ± 0.48 ($n = 3$) and 0.52 ± 0.12 n*M* ($n = 3$), respectively. The difference in K_d values is not statistically different ($p > 0.05$). These K_d values were within the range of K_d values for nuclear receptors and their HREs.

Binding of HIV-5,6 with high affinity to TR4 can also be demonstrated by the cold competition experiment. The equilibrium inhibition constant (K_i) for the specific binding of the labeled HIV-5,6 to TR4 as calculated from the cold competition experiments was 0.53 n*M*, which is similar to the K_d value obtained directly from the binding dose–response experiments (Fig. 2). For comparison, cold competition of one of the consensus direct repeats, DRI, was also performed. The K_i value of DRI (0.036 n*M*) was about 10-fold lower than that of HIV-5,6 (Fig. 3). This calculated

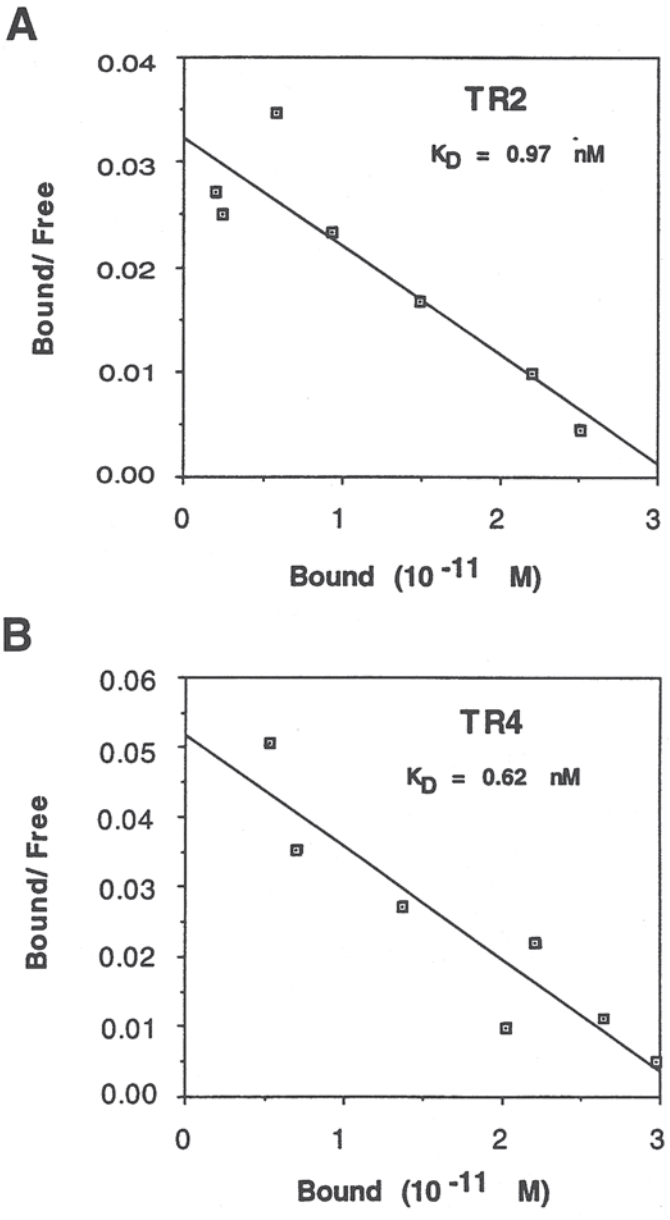

Fig. 2. Scatchard analysis of the binding of the ^{32}P-labeled probe (HIV-5,6, palindromic element) to orphan receptors TR2 (**A**) and TR4 (**B**), which were in vitro translated in a rabbit reticulocyte transcription translation system. TR2 and TR4 had roughly the same K_d value (0.97 and 0.62 n*M*, respectively). Increasing amounts of the ^{32}P-labeled probe were reacted with the in vitro translated orphan receptor, TR2 or TR4. Band shifts were developed and quantified by an ImageQuant software. To quantify the total probe in the reaction, an known amount of the probe was spotted onto a filter paper and then quantified by the ImageQuant software.

K_i value for DRI binding to TR4 is very close to the previously published K_d value for DRI binding to TR2 (K_d = 0.028 n*M*) *(15)*. Interestingly, each half-site of the palindromic element (HIV-3,4 as well as HIV-7,8) is sufficient to compete with the binding of the labeled palindromic element to TR4, although the equilibrium inhibition constant is higher.

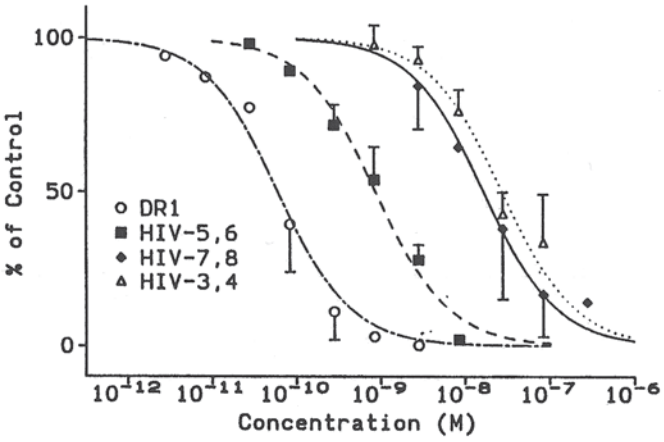

Fig. 3. Competitive inhibition of synthetic DRI, HIV-5,6, HIV-3,4, and HIV-7,8 on the binding of the ^{32}P-labeled palindromic element (HIV-5,6) to the in vitro translated TR4. For competition studies, a known concentration of the unlabeled probe (DRI, HIV-5,6, HIV-7,8, or HIV-3,4) was added to compete with the binding of a fixed amount of the ^{32}P-labeled HIV-5,6 probe (1 ng). After 30 min of incubation at room temperature, the bound and unbound ^{32}P-labeled palindromic element (HIV-5,6) was separated through a 5% native polyacrylamide gel at 4°C. Band shifts were developed and quantified by an ImageQuant software.

Effect of TR2 and TR4 on the Transcriptional Activity of HIV-LTR

To investigate whether TR2 and TR4 could regulate the gene expression by HIV-LTR, we carried out the chloramphenicol acetyl transferase (CAT) assay following the cotransfection of expression vectors and CAT reporter gene plasmids into Chinese hamster ovary (CHO) cells and QM7. Surprisingly, neither TR2 nor TR4 showed any significant induction or repression on the transcriptional activity of HIV-LTR with a dose up to 4 μg in either CHO or QM 7 cells (Fig. 4), although both TR2 and TR4 bind to the palindromic element in the HIV-LTR with extremely high affinities.

TR4 Potentiated the Transcriptional Induction of HIV-LTR by COUP-TF1 and TRα1

To explore further the above paradoxical results of TR2 and TR4 on the transcriptional regulation of HIV-LTR, we cotransfected TR2 or TR4 with either pSRh-COUP-TF1 or pCMD-hTRαl. COUP-TF1 has been previously demonstrated to be able to bind to this palindromic element with high affinity and to regulate the transcriptional activity of HIV-LTR in a variety of cell lines *(12–14)*. Since TR2 and TR4 would bind to the palindromic element without inducing promoter activity, it was reasonable to speculate that TR2 and TR4 could compete with COUP-TF1 for the binding sites in the HIV-LTR and that TR2 or TR4 may serve as an endogenous inhibitor of the COUP-TF1-induced transcriptional activation of HIV-LTR. Instead, TR2 showed no inhibitor effect, but TR4 synergistically potentiated the COUP-TF1 transcriptional induction in a dose-dependent

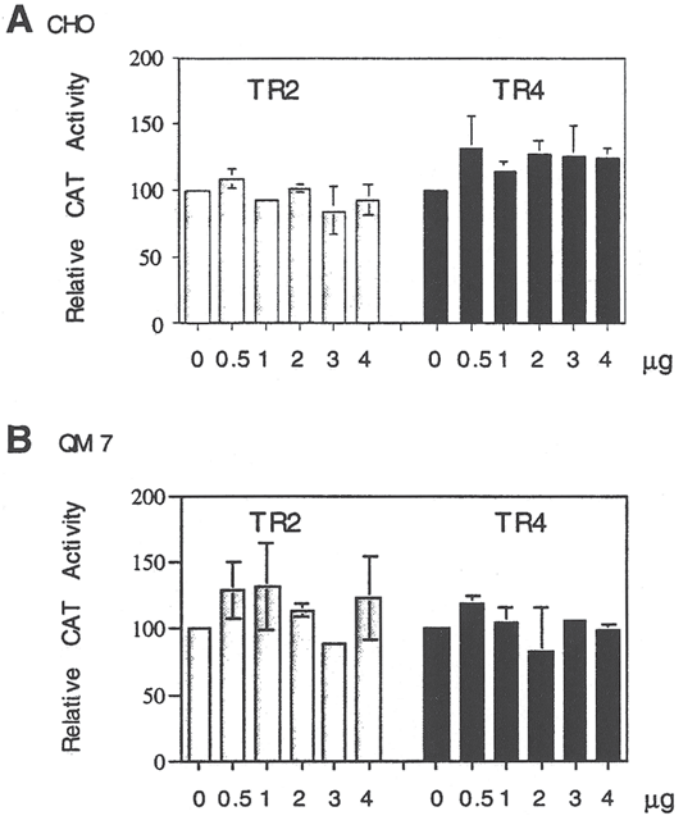

Fig. 4. Effect of TR2 and TR4 on the transcriptional activity of HIV-LTR. Neither TR2 nor TR4 showed induction or repression on the transcriptional activity of HIV-LTR with a dose up to 4 μg in either CHO cells or QM 7 cells. Cells were seeded in DMEM medium 24 h before transfection. The culture medium was replaced with a medium supplemented with charcoal dextran-treated FBS at 1 h before transfection. CHO **(A)** and QM7 **(B)** were cotransfected with a mixture of plasmids containing 2 μg of HIV-LTR with CAT reporter gene, 1 μg of β-galactosidase expression plasmids, and the indicated quantity of TR2 or TR4 receptor expression vector. The final amount of total DNA was brought up to 10.5 μg with the receptor cloning vector, pSG5 for TR2 and pCMX for TR4. Cells were harvested after 48 h of incubation at 37°C under 5% CO_2 for the determination of CAT activities. The β-galactosidase activity was used as the internal standard for the transfection efficiency. Each value represents the mean ± SD of 3–4 independent experiments.

manner (Fig. 5A). With cotransfection of 4 μg of TR4 and 1.5 μg of COUP-TF1 into QM7 cells, the CAT activity showed a further induction (two- to threefold) by TR4 as compared to the CAT activity resulting from the transfection of 1.5 μg of COUP-TF1 only (Fig. 5A).

In the absence of thyroid hormone, a unique TRα1 response element that overlaps with the Spl binding sites was identified in the core region of the HIV-LTR between bases –85 and –46. This TRα1 response element contains a single classical half-site AGGCCA on the noncoding strand between bases –66 and –71 and a sequence of GGCGGG on the coding strand between bases –64 and –59 *(16)*. Again, if TR2 and TR4 can bind specifically to the palindromic element in HIV-LTR, then TR2 and TR4 might

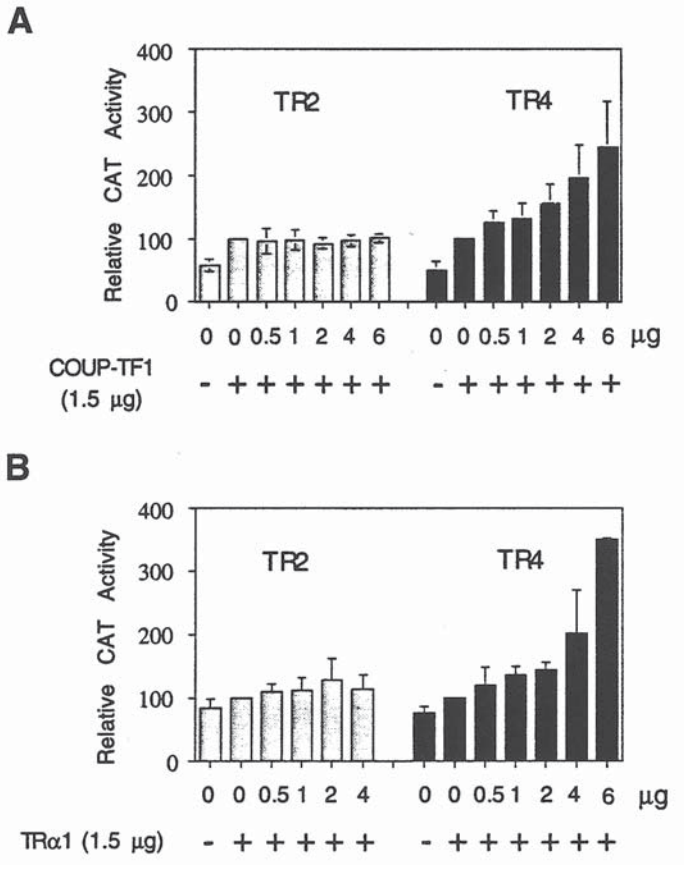

Fig. 5. Effects of TR2 and TR4 on the transcriptional activity of HIV-LTR induced by COUP-TF1 and TRα1. The experimental detail was similar to that described for Fig. 4. QM7 cells were cotransfected with a mixture of plasmids containing 2 μg HIV-LTR with CAT reporter gene, 1 μg of β-galactosidase expression plasmids, and the indicated quantity of receptor expression vector. 1.5 μg of pRS-hCOUP-TF1 plasmid or 1.5 μg of pCDM-hTRα1 receptor vector was added to the transfecting DNA mixture to express the human COUP-TF1 **(A)** or human TRα1 **(B)**. The final amount of total DNA was brought up to 10.5 μg with the receptor cloning vector, pSG5 for TR2 and pCMX for TR4. The β-galactosidase activity was used as the internal standard for the transfection efficiency. Each value represents the mean ± SD of 3–4 independent experiments.

have some effect on the transcription induction of HIV-LTR by TRα1. As expected, TR4 was able to induce synergistically with TRα1 in a dose-dependent manner (Fig. 5B). A two- to threefold induction in the CAT activity was seen in QM 7 cells when cells were cotransfected with TR4 and TRα1 (Fig. 5B). Again surprisingly, TR2 showed no effects on the transcriptional activity of HIV-LTR induced by TRα1.

To rule out the possibility that the nonresponsiveness of TR2 may be due to the defect in the TR2 plasmid, we have cotransfected both TR2 and TR4 with a plasmid CAT reporter gene containing a DR4 HRE, TK-$(DR4)_2$-CAT *(17)*. Both TR2 and TR4 induced the transcriptional activity of the CAT reporter gene containing the DR4 HRE (data not shown), proving the plasmid used to express TR2 in these experiments was functional.

Discussion

The AGGTCA motif has been shown to be the primary target element for the members of the estrogen response element (ERE) subfamily in the steroid-thyroid hormone receptor superfamily *(18)*. TR2 and TR4 bind to this core sequence with various affinities depending on the spacing between the two AGGTCA half-site direct repeat (DR) consensus sequences *(15,19)*. TR2 and TR4 have also been demonstrated to bind to several natural HREs and regulate their gene expression. These identified natural HREs include a DR1-type element in the cellular retinol binding protein II promoter (CRBPIIp) region, a DR2 type in the SV40 major late promoter in the +55 region, a DR5 type in the retinoic acid response element β (RAREβ), a DR1-type NGFI-β response element, and a DR4-type and a palindromic thyroid HRE *(15)*. These results suggest that TR2 or TR4 may bind to HREs overlapped with some other nuclear receptors, such as RAR, retinoid X receptor (RXR), vitamin D receptor (VDR), and TRα1.

However, so far there seem to be no rules to predict whether TR2 or TR4 will act as transcriptional activator or repressor. The key expression of the SV40 major late promoter could be repressed by the human TR2 and TR4 orphan receptors via the +55 region of the SV40 major late promoter *(9,10)*. TR2 orphan receptor was reported to bind to a DNA-response element to repress the human erythropoietin gene expression *(20)*. Also, the α component of the ciliary neurotrophic factor receptor (CNTFRα), with DR1 in its fifth intron, was identified to be the target gene that could be upregulated by TR4 *(6)*. Here, TR2 and TR4 function as neither an activator nor a repressor, although both could bind to this palindromic element with high affinity.

The molecular specificity of the steroid hormone receptors is achieved by their selective interaction with HREs *(21)*. The HREs are structurally related, but functionally distinct. Based on the finger model, the first zinc finger in the DNA binding domain of steroid hormone receptors may determine the target specificity of HRE. Five amino acids at the C-terminal region of the first zinc finger are marked as the proximal (P) box, which is important in base interaction *(18,21–23)*. TR2 and TR4 share identical amino acids in the P box and have a high homology in the DNA binding domain (81.2%). Therefore, both bind to the palindromic element with a similar affinity as demonstrated here in the retardation assay.

In the cold competition assay, either one of the half-sites of the palindromic element is sufficient to compete with the labeled palindromic element to bind to either TR2 or TR4, although a full length of the palindromic element will produce a tighter binding. In transiently transfected CAT assays, TR2 or TR4 alone show no effects on the transcriptional activity of HIV-LTR, but TR4 could couple to COUP-TF1 and TRα1 to enhance HIV-LTR transcriptional activity. It would be easier to explain our data if TR2 and

TR4 could form homodimers and if TR4, but not TR2, could form heterodimers with COUP-TF1 and TRα1. However, it has never been proven that TR2 and TR4 could form homodimers or heterodimers in the gel retardation assay *(9,10,19)*, although formation of homodimers and heterodimers between nuclear receptors are commonly observed *(24)*. It is clear that more work is needed.

As HIV continues to spread and as AIDS claims ever more lives, understanding this disease and devising therapies becomes an increasingly more urgent task. Further studies of how TR4 regulates HIV-1 may allow us to understand better the control of HIV-1 expression.

Materials and Methods

Materials and Construction of Plasmids

The plasmid pCMV-pGAL (β-galactosidase) was originally purchased from Clontech, Inc. (South San Francisco, CA). The plasmids pSG5-TR2 *(3)* and pCMX-TR4 *(10)*, which contain the full-length TR2 and TR4 cDNA, were previously constructed in our laboratory. The plasmid pGEM-7ZF(+) inserted with HIV-LTR and a CAT reporter gene (HIV-LTR-CAT) *(25)* was a gift of John A. A. Ladias, New England Deaconess Hospital, Harvard Medical School, Boston, MA. The Plasmids pRSh-COUP-TF1 *(13)* and pCD-hTRα1 *(26)*, were obtained from Ming-Jer Tsai and J. DeGroot, respectively. Oligonucleotides (DRI; HIV-3,4; HIV-5,6; and HIV-7,8) were synthesized at the Biotechnology Center, University of Wisconsin, Madison. Acetyl-CoA and the thin-layer chromatographic silica plates were obtained from Sigma (St. Louis, MO), and the ^{14}C-labeled chloramphenicol was from Amersham. All other chemicals are reagent grades.

Electrophoretic Mobility Shifting Assay (EMSA)

EMSA was performed according to the methods described by Cooney et al. *(13)*. In short, 100 ng double-stranded oligonucleotides corresponding to HIV-LTR palindromic element (5'AGGGGTCAGATATCCACTGACCTTT-3', HIV-5,6) were 5'-end-labeled with [γ^{32}P]ATP (DuPont NEN, Wilmington, DE) by using T4 polynucleotide kinase. The TR2 and TR4 orphan receptors were in vitro translated in a rabbit reticulocyte transcription translation system (Promega Co., Madison, WI). The in vitro translated receptor was incubated with the ^{32}P-labeled probe at room temperature for 30 min in an EMSA medium containing 10 m*M* HEPES (pH 7.9), 100 m*M* KCI, 1 m*M* dithiothreitol, 0.05 m*M* EDTA, 2.5 m*M* Mg_2Cl_2, 6% glycerol, and 2% Ficoll. For antibody supershift analysis, the antiserum from a rabbit immunized with TR2 or the anti-TR4 antibody (MAb #29) was incubated with the in vitro translation products at room temperature for 15 min, and then the probe was added. For competition reactions, unlabeled double-stranded oligonucleotides were mixed with the labeled probe, and then added to the reaction mixture. Protein–DNA complex and free oligonucleotide probe were separated by a 5% native polyacrylamide gel using a 0.5X Trisborate-EDTA (TBE) running buffer (1X TBE is 40 m*M* Tris borate and 1 m*M* EDTA, pH 8.0) at 150 V constant voltage at 4°C. Band shifts were developed and quantified by an ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). The IC_{50} value was defined as the competitor concentration needed to inhibit 50% of the binding of the labeled probe to TR4 and was obtained by the nonlinear regression with the GraphPad computer software. The K_i value was calculated from the Cheng and Prusoff equation *(27,28)*.

Cell Culture and Transient Transfection

CHO and QM 7 cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Inc., Grand Island, NY) with 100 U/mL penicillin G sodium and 100 μg/mL streptomycin sulfate and supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) (Bioproducts for Science, Inc., Indianapolis, IN). Cells were seeded in culture medium on 60-mm Petri dishes at a density of 1.5×10^5 cells/dish 24 h before transfection. One hour before transfection, the culture medium was replaced with 2 mL fresh experimental medium (DMEM supplemented with 5% charcoal dextran-treated fetal bovine serum) as previously described *(11)*. The transfection was performed with the modified calcium phosphate method *(29)*. The plasmid DNA was mixed with $CaCl_2$ to a final volume of 0.2 mL with a calcium concentration of 0.25 *M*, and then 0.2 mL of 2X BBS (50 m*M* BES, 280 m*M* NaCl, and 1.5 m*M* sodium bisphosphate, pH 6.98) was added dropwise while vortexing. After 15 min of incubation at room temperature, the calcium–phosphate–DNA mixture (400 μL) was added drop by drop to the plate of the cells while gently swirling the culture plate. The plasmid DNA contains 2 μg of HIV-LTR plasmids with a CAT reporter gene, 1 μg of β-galactosidase expression plasmids and the indicated quantity of receptor expression vector. The final DNA concentration was brought up to 10.5 μg with the receptor cloning vector, pSG5 for TR2 and pCMX for TR4. After 24 h of incubation at 37°C under 5% CO_2, the transfected medium was replaced with fresh experimental medium with or without hormone. The plasmid pCMV-βGAL was cotransfected as the internal standard, and the resulting β-galactosidase activity was used to normalize the transfection efficiency.

CAT Assay

Transfected cells were washed three times with phosphate-buffered saline (PBS), scraped into 1 mL of PBS, lysed by quickly freezing in ethanol with dry ice and thawing at 37°C in water, and then centrifuged at 14,000*g* for 2 min. The β-galactosidase activity was measured from 15 μL of supernatant, by incubating at 37°C for 15–30 min in 0.1 *M* Na phosphate buffer (pH 7.5) with 0.88 mg

o-nitrophenyl-β-D-galactopyranoside in a final volume of 150 μL. The reaction was stopped with 250 μL of 1 *M* Na_2C0_3, and the amount of *o*-nitrophenol formed was measured spectrophotometrically at 420 nm. The β-galactosidase activity was used as the internal standard for the transfection efficiency to determine the proper aliquot of 14,000*g* supernatant to be used for the CAT activity measurement. The lysates prepared from the transfected cells and used in the CAT assay were heated at 60°C for 10 min to inactivate endogenous deacetylase activity *(30)*. CAT activities were determined by incubating the heat-inactivated cell lysates with 30 μg acetyl-CoA and 0.9 n*M* ^{14}C-labeled chloramphenicol (50 nCi) at 37°C. Acetylated and unacetylated forms of this compound are extracted with ethyl acetate, concentrated, and then separated by a silica TLC plate, which was run in a 95% chloroform and 5% methanol solvent. The unreacted ^{14}C-labeled chloramphenicol and the reaction products (both the mono- and diacetylated chloramphenicols) were quantified by an ImageQuant software.

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References

1. Evans, R. M. (1988). *Science* **240,** 889–895.
2. Beato, M. (1989). *Cell* **56,** 335–344.
3. Chang, C., Kokontis, J., Acakpo-Satchivi, L., Liao, S., Takeda, H., and Chang, Y. (1989). *Biochem. Biophys. Res. Commun.* **165,** 735–741.
4. Chang, C., Da Silva, S. L., Ideta, R., Lee, Y., Yeh, S., and Burbach, J. P. H. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 6040–6044.
5. Young, W.-J. (1996). PhD Thesis, University of Wisconsin, Madison.
6. Young, W.-J., Smith, S., and Chang, C. (1997). *J. Biol. Chem.* **272,** 3109–3116.
7. Lin, D.-L. and Chang, C. (1996). *J. Biol. Chem.* **271,** 14,649–14,652.
8. Fauci, A. S. (1996). *Nature* **384,** 529–534.
9. Lee, H.-J. and Chang, C. (1995). *J. Biol. Chem.* **270,** 5434–5440.
10. Lee, H.-J., Lee, Y., Burbach, J. P., and Chang, C. (1995). *J. Biol. Chem.* **270,** 30,129–30,133.
11. Lin, D.-L. (1996). PhD Thesis, University of Wisconsin, Madison.
12. Cooney, A. J., Tsai, S. Y., O'Malley, B. W., and Tsai, M. J. (1991) *J. Virol.* **65,** 2853–2860.
13. Cooney, A. J., Tsai, S. Y., O'Malley, B. W., and Tsai, M. J. (1992). *Mol. Cell. Biol.* **12,** 4153–4163.
14. Sawaya, B. E., Rohr, O., Aunis, D., and Schaeffer, E. (1996). *J. Biol. Chem.* **271,** 23,572–23,576.
15. Lin, T.-M., Young, W.-J., and Chang, C. (1995). *J. Biol. Chem.* **270,** 30,121–30,128.
16. Rahrnan, A., Esmaili, A., and Saatcioglu, F. (1995). *J. Biol. Chem.* **270,** 31,059–31,064.
17. Lee, Y.-F, Pan, H.-J., Burbach, J. P. H., Morkin, E., and Chang, C. (1997). *J. Biol. Chem.* **272,** 1–6.
18. Mader, S., Kurnar, V., de Verneuil, H., and Charnbon, P. (1989). *Nature* **338,** 271–274.
19. Lee, Y.-F. (1997). PhD Thesis, University of Wisconsin, Madison.
20. Lee, H.-J., Young, W.-J., Shih, C.-Y., and Chang, C. (1996). *J. Biol. Chem.* **271,** 10,405–10,412.
21. Umesono, K. and Evans, R. M. (1989). *Cell* **57,** 1139–1146.
22. Danielsen, M., Hinck, L., and Ringold, G. M. (1989). *Cell* **57,** 1131–1138.
23. Nelson, C. C., Hendy, S. C., Fairs, J. S., and Romaniuk, P. J. (1994). *Mol. Endocrinol.* **18,** 829–840.
24. Perlmann, T. and Evans, R. M. (1997). *Cell* **90,** 391–397.
25. Ladias, J. A. A. (1995). *J. Biol. Chem.* **269,** 5944–5951.
26. Nakai, A., Sakurai, A., Macchia, E., Fang, V., and CeGroot, L. J. (1990). *Mol. Cell. Endocrinol.* **72,** 143–148.
27. Cheng, Y. and Prusoff, W. H. (1973). *Biochem. Pharmacol.* **22,** 3099–3104.
28. Hwang, S.-B., Lam, M.-H., BiRu, T., Beattie, T. R, and Shen, T. Y. (1985). *J. Biol. Chem.* **260,** 15,639–15,645.
29. Chen, C. and Okayama, H. (1987). *Mol. Cell. Biol.* **7,** 2745–2752.
30. Ginot, R., Decaux, J.-F., Cognet, M., Berbar, T., Levrat, F., Kahn, A., et al. (1989). *Eur. J. Biochem.* **180,** 289–294.