Multiple Functions of the TR2-11 Orphan Receptor in Modulating Activation of Two Key Cis-acting Elements Involved in the Retinoic Acid Signal Transduction System

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The testicular receptor 2 (TR2) orphan receptor binds to hormone response elements (HREs) consisting of two AGGTCA half-site direct repeat consensus sequences (DR) with various spacing in the following order: DR1 > DR2 > DR5 DR4 DR6 > DR3. When binding to natural HREs, TR2 orphan receptor remains flexible with higher binding affinities to (a) cellular retinol-binding protein II promoter region (CRBPIIp) (DR1), SV40 (55 region (DR2), and retinoic acid response element β (RAREβ) (DR5) than to (b) NGFI-B response element (NBRE) and also to (c) the palindromic thyroid hormone response element (TREpal). This wide spectrum of HRE recognition sequences suggests possible versatility of the TR2 orphan receptor in cross-talking with other signal transduction systems. Chloramphenicol acetyltransferase (CAT) assay demonstrates that the TR2 orphan receptor competes with CRBPIIp- and RAREβ-CAT gene expression activated by retinoid X receptor α (RXRα) and retinoic acid receptor α (RARα)/RXRα heterodimers, respectively. In addition, this suppression may not be mediated by the formation of heterodimers between TR2 orphan receptor and either RXRα or RARα. Instead, a minimum of 100-fold higher affinity of the TR2 orphan receptor for CRBPIIp than RXRα-RARα may explain why the TR2 orphan receptor dominates RXRα in CRBPIIp-CAT activation. Together, our data suggest that the TR2 orphan receptor may be a master regulator in modulating the activation of two key HREs, RAREβ and CRBPIIp, involved in the retinoic acid signal transduction pathway.

Sex steroids, adrenal steroids, thyroid hormones, vitamin A and D derivatives in vertebrates and ecdysone in Drosophila play key roles in triggering tissue and embryo development. Their corresponding intracellular receptors have been revealed to be similar and belong to a steroid receptor superfamily (1). These ligands bind to their specific receptors and the ligand-receptor complex then interacts with cis-acting DNA hormone response elements (HREs)1 found mostly at the 5′ promoter region of the target genes. As a result, the transcription of these specific target genes is either activated or suppressed (2–4). In addition to those receptors involved in mediating the specific ligand signal transduction systems, a large proportion of this gene superfamily consists of putative receptors, named orphan receptors, having an unidentified ligand (5).

TR2 orphan receptor has been isolated from human prostate and testis cDNA libraries with a probe designed to select clones encoding the steroid receptor DNA binding domain. Three TR2 subclasses, TR2-5, TR2-7, and TR2-9, were isolated from a human testis library; while the fourth clone, TR2-11, was identified in a human prostate gt11 cDNA library (6). An identical DNA binding domain suggests that all TR2 orphan receptor subtypes may act on the same target HREs. Consequently, we have selected TR2-11 as the starting point to investigate the function of TR2 orphan receptors. The TR2-11 orphan receptor is a protein of 603 amino acids with a calculated molecular mass of 67 kDa. The TR2 orphan receptor has been expressed in many rat tissues with higher abundance in male reproductive organs (7). The transcription of TR2 orphan receptor mRNA is negatively regulated by androgen in the human prostate LNCaP cell line and rat ventral prostate.

Based on the amino acid sequences, all members of the steroid receptor family share a high conservation in the DNA binding domain which is predicted to form two zinc finger motifs. The first zinc finger, containing the P box sequences, is proposed to be essential for the recognition of a specific response element, while the second one is involved possibly in protein-protein interactions, e.g. dimerization with receptors among the same family (1, 8). Consequently, these receptors can be grouped into either the estrogen receptor (ER) or the glucocorticoid receptor subfamily. The ER subfamily consists of ER, thyroid hormone receptors (TRs), retinoic acid receptors (RARs), retinoid X receptors (RXRs), vitamin D receptor, and many orphan receptors, including the TR2 orphan receptor (9).

They all have a unique glutamic acid residue following the third cysteine residue found in the P box, and they are predicted to recognize palindrome estrogen response element (ERE)/TRE (AGGTCA (n), TGACCT, x = 0 or 3) sequences (10–13). Lately, investigators have demonstrated that the some of the ER subfamily members also recognize AGGTCA in a direct-repeat (DR) orientation (AGGTCA (n), AGGTCA, x = 0–6) or even an AGGTCA hexamer half-site with additional nucleotides at 5′ end (ln), AGGTCA). For instance, RXRs form heterodimers with vitamin D receptor, TRs, andRARs upon response element; CAT, chloramphenicol acetyltransferase; RXR, retinoid X receptor; ER, estrogen receptor; TR, thyroid hormone receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; SV40, simian virus 40; TRE, thyroid hormone response element; RBA, relative binding affinity; TFA, all-trans-retinoic acid; 9cRA, 9-cis-retinoic acid; CRABP, cellular retinoic acid binding protein.

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1. The abbreviations used are: HRE, hormone response element; DR, direct-repeat consensus response element; EMSA, electrophoretic mobility shift assay; CRBPII, cellular retinol binding protein II; RAR, retinoic acid receptor; RARE, retinoic acid response element; NBRE, NGFI-B response element; TREpal, palindromic thyroid hormone response element; CAT, chloramphenicol acetyltransferase; RXR, retinoid X receptor; ER, estrogen receptor; TR, thyroid hormone receptor; COUP-TF, chicken ovalbumin upstream promoter transcription factor; SV40, simian virus 40; TRE, thyroid hormone response element; RBA, relative binding affinity; TFA, all-trans-retinoic acid; 9cRA, 9-cis-retinoic acid; CRABP, cellular retinoic acid binding protein.

30121
the recognition and activation of response elements consisting of AGGTCA half-site tandem direct repeats with 3-4 base spacing, respectively (14). Chicken ovalbumin upstream promoter transcription factor (COUP-TF) has been shown to recognize not only AGGTCA direct repeats with various spacing but also AGGTCA palindromic elements (15). NGF1-B prefer an octameric HRE, AAGGCTA, and functions as a mononmeric trans-acting receptor (16).

Here we demonstrate that TR2 orphan receptor recognizes not only AGGTCA direct repeats with various spacing but also TRE palindromic repeat, albeit at a much lower affinity. The flexibility of TR2 orphan receptor may allow it to form monomeric binding with single half-site HRE, e.g. NBRE. With the emphasis on the retinoic acid signal transduction system, we demonstrate that TR2 orphan receptor binds to RAREβ and CRBP1p HREs with high affinities in vitro and competes for CAT reporter gene expression in cell culture with high efficiency.

MATERIALS AND METHODS

Construction of Plasmids—The RAREβ-pCtAP and CRBP1p-pCtAP reporter plasmids were constructed as follows. Double-stranded RAREβ and CRBP1p oligonucleotides (sequences shown in Table I) were inserted into the BglII digestion site located at the 5′ end of an SV40 basic promoter in the pCtAP vector promoter (Promega Co., Madison, WI). The final constructs were confirmed by DNA sequencing (CircumVent thermal cycle sequencing kit, New England Biolabs, Inc., Beverly, MA) to ensure only one copy of RAREβ or CRBP1p is inserted in the right orientation.

The pSgS-TR2-ARP-TR2 expression vector was designed to swap the hAR P-box sequence into the corresponding TR2 orphan receptor coding region. A 5′ end polymerase chain reaction primer, TCAGGACGTCATGAGAGACTGAATCCGCAATTCCTGTGAAGCAGTAACTTGTGGAA*GCTGCAAAGT*C*T-AGGTCA direct repeats with various spacing, CRBP1p HREs with high affinities in vitro and competes for CAT reporter gene expression in cell culture with high efficiency.

RESULTS

TR2 Orphan Receptor Binds to Consensus DRs with Different Affinities—We have tested the preference of the TR2 orphan receptor for binding to consensus AGGTCA tandem repeats with 1- to 6-base pair spacing (DR1 to DR6), sequences shown in Table I, by means of cold competitive EMSA analyses. Specific protein-DNA band shift was identified by comparing EMSA profiles obtained from experiments done with expression vector conditioned reticulocyte lysate and those done with nonconditioned lysate (mock). After EMSA specific band shifts (shown in Fig. 1) were quantified, the 50% competition concentrations (IC50) and the RBAs were calculated, as described under “Materials and Methods” and listed in Table I. Different RBAs between various DRs demonstrate clearly that the TR2 orphan receptor can interact with perfect AGGTCA DR type HREs with a preference in the order of DR1 (RBA = 1.00) > DR2 (RBA = 0.56) > DR5 (RBA = 0.28) > DR4 (RBA = 0.24) > DR6 (RBA = 0.21) > DR3 (RBA = 0.077).

TR2 Orphan Receptor Binds to Natural HREs in Great Varities—To search for the potential natural TR2 HREs, several natural HREs (sequences shown in Table I) were synthesized and tested for binding to the TR2 orphan receptor. An RXR response element, CRBP1p sequence is a DR1 type element (20). A DR2 type HRE was identified in SV40 late promoter in the +55 region which is protected from DNase I digestion by initiator-binding protein (21). RAREβ, a DR5 type HRE, has been demonstrated to respond to the activation of RARα/RXRα heterodimer upon the addition of retinoic acid (RA).

Cold competitive EMSA experiments were performed and shown as Fig. 2A. The band shift intensity versus concentration of competitor results were plotted out as Fig. 2B and RBAs calculated as listed in Table I. These RBA data reveal that the
affinities of TR2 orphan receptor to natural HREs are lower than those to perfect consensus HREs (e.g., comparing RBA of DR1 (4.19 ± 1.00) versus that of CRBP II p (1.00)). And the binding preference of TR2 orphan receptor to these natural HREs also follows the order of DR1 > DR2 > DR5: TR2 orphan receptor has the highest affinity to DR1 type HRE, i.e., CRBP II p (1.00), which is followed by DR2, SV40 + 55 (RBA = 0.104 ± 0.003), and then by DR5, RARE β (RBA = 0.0101 ± 0.0029).

The RBA values of RARE α, β, and γ are 1.00, 0.0101, and 0.0006, respectively. Therefore, the binding of RAREs to their HREs is much weaker than that of the orphan receptor to natural HREs. The binding preferences of TR2 orphan receptor to conserved perfect direct repeat HREs were determined by cold competitive EMSA. In Table I, we have applied the EMSA analyses to study the potential natural TR2 orphan receptor HREs identified by EMSA analyses, are cis-acting regulatory elements for RAR and CRBP II genes, respectively. To investigate whether TR2 orphan receptor can interfere in the formation of RAR/RXR heterodimer, not RAR or RXR homodimer, shifted RARE β to a position different from that of the TR2 orphan receptor band shift. Taking advantage of the significant distance between the band shifts, we also tested whether TR2 orphan receptor can interfere in the formation of RAR/RXR heterodimer by forming a heterodimer with either RAR α or RXR α in the presence of RARE β HRE. The results, shown in Fig. 3A, demonstrate a clear band shift when TR2 orphan receptor is incubated with 32P-labeled probe to compete nonspecifically and effectively with 32P-labeled probe to bind TR2 orphan receptor.

Fig. 1. Binding preference of TR2 orphan receptor to consensus perfect direct repeat HREs. Cold competitive EMSA analysis was performed with 0.1 ng of 32P end-labeled DR1 oligomer, 1 μl of in vitro translated TR2 orphan receptor, and applicable cold competitive oligomer as marked (DR1-DR6). Five quantities (0.00625, 0.025, 0.1, 0.4, and 1.6 ng) of each consensus direct repeat HRE, sequences listed in Table I, were allowed to compete for binding to TR2 orphan receptor. The specific band shifts were quantitated with PhosphorImager; the I50 concentrations were determined and RBAs calculated as described under “Materials and Methods.” The RBAs of these DRs to TR2 orphan receptor are listed in Table I. Data are shown as a representative autoradiogram of two independent experiments.
caused a diminishment of TR2 orphan receptor band shift intensity (compare lanes 1 and 5 in Fig. 3B). These results suggest strongly that there is a competition between TR2 orphan receptor and RARα/RXRα heterodimer upon binding to RAREβ motif; however, TR2 orphan receptor may not directly interact with either RARα or RXRα by forming heterodimers.

TR2 Orphan Receptor and RARα/RXRα Heterodimer Bind to RAREβ with Similar Affinities—To compare binding affinities between TR2 orphan receptor and RARα/RXRα to RAREβ HRE, saturation Scatchard analyses were performed as described under “Materials and Methods.” Fig. 4, A and B, show typical patterns of protein-DNA complex formed between the increasing amounts of RAREβ oligomers (from 0.0625 to 8 ng in a total 20-μl reaction volume) and fixed amounts of TR2 orphan receptor and RARα/RXRα, respectively. Specific complex (bound) intensities were quantitated and plotted against unbound (free) probes as saturation curves, illustrated in Fig. 4C. The consequent Scatchard analysis was performed and calculated dissociation constants (Kd) for binding of TR2 orphan receptor and RARα/RXRα heterodimer to RAREβ are 5.03 and 2.32 nM, respectively (Fig. 4C, inset). The comparable Kd values between these two groups of receptors suggest that TR2 orphan receptor may also express some transcriptional regulatory effects via binding with the RAREβ cis-acting element.

TR2 Orphan Receptor Down-regulates the Expression of a CAT Reporter Construct Carrying One Copy of RAREβ—To test this possibility, we have constructed an RAREβ-pCATp reporter plasmid. The effect of endogenous RARα/RXRα on RAREβ-pCATp expression was analyzed with CV1 cells which were transiently transfected with RAREβ-pCATp construct only and exposed to various concentrations of all-trans-retinoic acid (tRA), which may function as an RARα ligand and also as an RXRα activator (20, 25-27). As shown in Fig. 5, with increasing concentrations of tRA, CAT activities increased up to 6.7-fold in a dose-dependent fashion (Fig. 5, lanes 1 -3). In the absence of RA, the co-transfected pSG5-TR2 expression vector caused a 10-fold decrease in CAT activity (compare Fig. 5, lane 1 versus 4). Even in the presence of tRA, significant decreases in CAT activities resulted from co-transfected pSG5-TR2 are
The stimulatory effects of endogenous RAR with similar affinities. Scatchard analyses demonstrate that at least a 100-fold difference in affinity exists between TR2 orphan receptor and RARα. Since our data showed that TR2 orphan receptor has the highest affinity to CRBPⅠp among tested natural HREs, we are also interested in the binding affinities of TR2 orphan receptor. Scatchard analyses demonstrate that at least a 100-fold difference in affinity exists between TR2 orphan receptor (Kd = 0.028 nM) and RARα (Kd = 4.36 nM) in binding to CRBPⅠp in vitro (Fig. 6B). The addition of RARα ligand, 10−8 M 9-cis-retinoic acid, has failed to enhance affinity between RARα and CRBPⅠp (Fig. 6B, inset).

TR2 Orphan Receptor Competes with RARα in Activating the Same CRBPⅠp cis-acting Element—To determine whether the interaction between TR2 orphan receptor and CRBPⅠp may result in possible transacting activities in vivo, we have constructed a CRBPⅠp-pCATp reporter plasmid. In the presence of 10−6 M tRA, an RARα activator, our results showed no significant increase in CAT activity obtained from CV1 cells transfected only with CRBPⅠp-pCATp (data not shown). However, tRA could induce CAT activities up to 11.5-fold when CV1 cells were co-transfected with CRBPⅠp-pCATp and pCMX-rHⅠRα expression vectors (Fig. 7, lanes 1 and 3–5). In addition, the increase of CAT activity induced by exogenous RARα can be suppressed (from 11.5- to 3.5-fold increases of TR2 orphan receptor basal induction activity) by co-transfected pSG5-TR2 expression vector even in the presence of 10−6 M tRA (Fig. 7, lane 5 versus 8). These results suggest that the high binding affinity of exogenous RARα to CRBPⅠp may dominate the effect of RXRα on the expression of transfected RAREβ-CAT constructs. The exogenous TR2 orphan receptor can effectively compete for RAREβ-CAT activation even in the presence of tRA at a concentration of 10−6 M.

TR2 Orphan Receptor Expresses during Late Fetal Development—Since our data showed that TR2 orphan receptor is expressed in many tissues, including kidney and intestine, when the mouse expresses in these two organs. As shown in Fig. 8, our results show that the inhibitory effect of the TR2 orphan receptor on the exogenous RARα basal induction activity by co-transfected pSG5-TR2 expression vector is not due to any titration of co-activator, we have constructed a mutated TR2 orphan receptor, pSG5-TR2-ARp-TR2. This mutated TR2 expression vector has been generated by swapping TR2 orphan receptor P-box with that found in human androgen receptor gene. As a result, this mutated TR2 orphan receptor may reserve all the properties of wild type TR2 orphan receptor except the binding affinity to CRBPⅠp HRE. The Kd changes from 0.028 nM to 1.44 μM (data not shown) support this argument. Furthermore, the CAT reporter gene assay, shown in Fig. 7, lanes 9–11, demonstrates that this mutated TR2 orphan receptor cannot effectively repress CAT activities induced by RARα in the presence of tRA. Together, these results show that the inhibitory effect of the TR2 orphan receptor on CRBPⅠp requires direct receptor HRE interaction.

TR2 Orphan Receptor Expresses during Late Fetal Development—Using in situ hybridization techniques, investigators were able to localize RARα/RARα in many tissues with higher expression in kidney and intestine (28–30). We, therefore, are also interested to see whether TR2 orphan receptor expresses in these two organs. As shown in Fig. 8, our results clearly demonstrated that TR2 orphan receptor expresses in many tissues, including kidney and intestine, when the mouse...
TR2 Orphan Receptor in Retinoid Responsive System

embryo is at the age of gestation 16.5 day. This co-expression of TR2 orphan receptor and RXRα/RXRα in kidney and intestine provides a strong argument supporting the possible role of TR2 orphan receptor in RXRα/RXRα signal pathway.

DISCUSSION

The biological significance of the human TR2 orphan receptor subfamily was not clearly demonstrated shortly after its identification (6, 7). The distribution of TR2 orphan receptor in many tissues was recently reconfirmed by in situ hybridization approaches done in mouse fetus. Also, recent studies have manifested the importance of this orphan receptor subfamily. The TR2 orphan receptor family has now expanded to include the TR4 orphan receptor (31), and the spatial difference in distribution among the members suggests an important role of the TR2 orphan receptor in physiological events. It has been demonstrated recently that the TR2 orphan receptor may repress transcription activities of the SV40 major late promoter (22). In prostate, the expression of TR2 orphan receptor can also be repressed by androgens (32). The identification of potential HREs that are recognized by the TR2 orphan receptor may expand our understanding of the function of the TR2 orphan receptor.

From amino acid and nucleotide sequences encoding the P-box of the DNA binding domain, one can place TR2 orphan receptor into the ER subfamily of the steroid receptor superfamily. Like other members in the ER subfamily, we have demonstrated in this paper that TR2 orphan receptor binds to all tested consensus HREs with the following preferences in terms of nucleotide spacing: DR1 > DR2 > DR5 DR4 DR6 > DR3. Three natural HREs that bind to the TR2 orphan receptor effectively were also identified: CRBPIIp (an RXRE), SV40 +55 region, and RAREβ (an RXRα/RXRα RE), respectively. In accordance with the RBAs of consensus HREs, these three potential TR2 HREs followed the same 1 > 2 > 3 space order in the RBAs.

Thyroid hormone receptors are known to bind as homodimers and heterodimers with RXRα to response elements consisting of AGGTCA hexamer half-sites in direct repeat, palindromic, and inverted palindromic orientations (14, 33–35). Similar to NGFI-B and FTZ-F1, thyroid hormone receptor has also been demonstrated to recognize octameric half-site HREs, NNAGGTCA consensus, and function as a monomeric transacting factor (36). Here we also demonstrate that NBRE,

Fig. 6. TR2 orphan receptor has higher affinity binding to CRBPIIp HRE than RXRα does. Saturation Scatchard analysis was performed according to data obtained from an EMSA done with increasing amounts of 32P-labeled CRBPIIp oligomers (0.0078, 0.031, 0.125, 0.5, and 2 ng) and fixed amounts of in vitro translated TR2 orphan receptor (0.1 µl). The dissociation constant (Kd), determined as the minus reciprocal of the Scatchard plot slope, between TR2 orphan receptor and CRBPIIp was calculated to be 0.028 nM (open square). While much higher Kd value between RXRα and CRBPIIp were determined as 4.36 nM (open circle). Additional RXRα ligand, 10–8 M 9cRA, altered the Kd to 4.83 nM (open triangle). Due to lesser affinity between RXRα and CRBPIIp, the Scatchard plots were constructed from EMSA data done with 32P-labeled CRBPIIp in amounts of 0.0625, 0.125, 0.25, 1, 2, 4, and 8 ng and 2 µl of in vitro translated RXRα.

Fig. 7. TR2 orphan receptor dominates over RXRα in regulating the expression of a CRBPIIp-pCATp construct. A, shows CAT reporter gene assay performed with CV1 cells. CV1 cells were transfected with CRBPIIp-pCATp reporter gene only (lane 1) or further cotransfected with pSG5 TR2 expression vector (lanes 3–8) and/or pCMX RXRα expression vector (lanes 2 and 6–8) in equal amounts (3 µg) as indicated. The CAT activities were induced by the addition of all-trans-retinoic acid (tRA) in concentrations of 10–9 M (lanes 4 and 7) and 10–6 M (lanes 5 and 8). B shows independent CAT reporter gene assay performed with CV1 cells. CV1 cells were transfected the same as A. Chloramphenical conversion rates were calculated from PhosphorImager quantifiable intensities. Fold inductions were normalized according to the chloramphenical conversion rate obtained from CV1 cells transfected with CRBPIIp-pCATp construct, without further added tRA (lane 1). Data shown here represents three independent experiments.

In the EMSA experiment, the Scatchard plots were constructed from EMSA data done with 32P-labeled CRBPIIp in amounts of 0.0625, 0.125, 0.25, 1, 2, 4, and 8 ng and 2 µl of in vitro translated RXRα.

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an AAAGGTCA half-site HRE, with an RBA double that of RAREβ competes with CRBP1p in binding to TR2 orphan receptor. However, whether this half-site HRE operates as a TR2 response element remains to be elucidated by at least a reporter assay in cell culture. On the other hand, to show effective competition against CRBP1p binding to the TR2 orphan receptor, approximately 140 times more concentrated TREpal is required, suggesting that TR2 orphan receptor binds to palindromic HRE weakly. However, whether or not palindromic repeats with various spacing alters the efficiencies in the competition has not been tested yet. Thus far we have not yet explored any putative TR2 HREs in an inverted palindromic orientation.

Study of HREs sequences has revealed that the TR2 orphan receptor recognizes various naturally available imperfect half-site sequences like AGTGTC (i.e. CRBP1p and RAREβ), AGGTCA (i.e. SV40 +55), and AGtTC (i.e. RAREβ). These findings indicate that TR2 orphan receptor has great flexibility in recognizing various DNA sequences, and thus significant protein conformational changes may be required to accommodate this phenomena. However, just one nucleotide change of the second G in AGGTTC/AGGTCA, into c, in AGGtTC/AGGtCA, in both half-sites of SV40 +55 abolishes the competitive ability completely (compare RBAs of SV40 +55 and SV40 +55 in Table 1). This result provides indirect evidence that the third position of the hexamer half-site may be a key determinant on which the TR2 orphan receptor relies for binding. Protein crystallographic or nuclear magnetic resonance approaches may provide further direct evidence of this flexibility (37). These results support the idea that TR2 orphan receptor may interact with a broad range of HREs involved in various signal transduction pathways.

The data presented here also suggest an important biological role of TR2 orphan receptors in retinoid response pathways. The retinoic acids (RAs) and retinoids are key elements in regulating embryogenesis, tissue differentiation, and teratogenesis. Several gene products have been demonstrated to be involved in this signal transduction system directly. For example, nuclear RARs and RXRs can be activated by RAs and operate as trans-acting factors that result in direct target gene transcription activation. Two groups of cytoplasmic proteins, cellular retinoic acid- and retinol-binding proteins (CRABPs and CRBPs, respectively) were characterized to bind with RAs and retinoids and may be involved in fine-tuning the concentrations of free intracellular RAs. Functionally, RA can cross-talk with various signal transduction pathways. RXRs are effective co-activators for vitamin D3, thyroid hormone, and retinoic acid receptors by forming stable heterodimers and thus can promote a greater level of gene activation (38).

In this report we demonstrate clearly that the TR2 orphan receptor down-regulates the stimulatory effect of RARα/RXRα on RAREβ CAT expression from monkey kidney CV1 cells in the presence of tRA. These results suggest a possibility that this TR2 orphan receptor is a strong suppressor for RAREβ activation and may result in the interruption of the RA gene regulation cascade. The tissue distribution and spatial expression pattern of RARα and RXRα gene during mouse fetal development are well documented using an in situ hybridization technique (28–30). Comparing these results and ours in Fig. 8, we conclude that TR2 orphan receptor, RARα, and RXRα are expressed abundantly in at least kidney and intestine during mouse embryo development. All these expression patterns from in situ hybridization experiments may manifest the potential physiological significance of this study.

COUP-TF orphan receptors have also been demonstrated to be negative regulators of the RA response pathways (39). Lately, this negative regulatory effect of COUP-TF has been expanded to vitamin D and thyroid hormone responding systems (15, 24). COUP-TF may activate the silencing of transcription by competing for cis-acting response elements and/or forming heterodimer with RXRα (15). Unlike COUP-TF, our EMSA experiments do not support the possibility that TR2 orphan receptor forms heterodimers with either RARα or RXRα receptors directly. Our observations shown here favor the hypothesis that this suppressive effect results from competition between TR2 orphan receptor and RARα/RXRα heterodimer by recognizing the same RAREβ sequences. RAREβ bound with TR2 orphan receptor may fail to activate the expression of RAREβ gene.

It is worth noting that TR2 orphan receptor showed at least 100-fold higher affinity in binding to CRBP1p response element than RXRα. Furthermore, CAT activities driven by the interaction between RXRα and CRBP1p could be suppressed when exogenous TR2 orphan receptors were made available. This high affinity of TR2 orphan receptor to CRBP1p-CAT con-
tructs could be occupied completely by exogenous TR2 orphan receptor and CAT activities driven accordingly. This argument is strengthened further by the observation that a P-box-mutated TR2 orphan receptor has failed to bind to CRBPII-mediated induction. In summary, our data suggest that TR2 orphan receptor may offer another regulatory mechanism involved in the RA signal transduction system.

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