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Published in Gen Comp Endocrinol. 2003 Jul;132(3):474-84. PMID: 12849971. DOI: 10.1016/S0016-6480(03)00116-3

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Running title: An orphan nuclear hormone receptor TRR

Keywords: TRR, TR2, TR4, orphan nuclear hormone receptors, gene expression.

[1] This work was supported by KOSEF through Hormone Research Center grant HRC-1999-L0001 (to H.-S. C.), Swiss National Foundation grant (to U. A. M.) and KOSEF grant R01-1999-00092 (to W.-S. K.)

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ABSTRACT

Orphan receptors belong to the nuclear receptor superfamily of liganded transcription factors, whose ligands either do not exist or remain to be identified. We report here the cloning and characterization of the novel avian orphan receptor TRR (for testis specific receptor 2 (TR2) related receptor). The TRR gene encodes a protein of 569 amino acids which shows approximately 72% overall identity with TR2 and 95% identity in the DNA binding domain (DBD). The TRR gene is expressed in almost all adult tissues and embryonic stages examined unlike its mammalian relative TR2, which is specifically expressed in testis. Electrophoretic mobility shift assays demonstrate that TRR binds the canonical direct repeat DNA recognition sequences spaced by one, four and five nucleotides (DR1, DR4, DR5), and in consistence with the results with canonical DNA binding sequences, TRR forms specific DNA-protein complex with chicken phenobarbital response elements containing DR4 motifs. Both *in vitro* and *in vivo* interaction studies demonstrate that TRR forms homodimer. Finally, transient transfection studies reveal its capability to transactivate canonical DR1, DR4 and DR5 sequences and the constitutive activity of TRR is mapped to the N-terminal region of this orphan receptor.

INTRODUCTION

The steroid/thyroid hormone receptor superfamily is a large group of related transcriptional factors that control cellular differentiation, development, and homeostasis by direct interaction with distinct *cis*-elements on their target genes. This superfamily includes receptors for steroids, thyroid hormone, vitamin D_3, retinoids and a large number of orphan receptors whose cognate ligands either do not exist or are still unknown (1). These proteins are able to bind to specific DNA sequences named hormone response elements (HREs) associated with their target genes. Sequence analysis and functional studies reveal a modular structure for these proteins (2). The N-terminal hypervariable region called A/B domain contains a ligand independent activation function AF-1 (activating function-1). The strongly conserved DNA binding domain (DBD), called C domain, is composed of two zinc finger motifs. The C-terminal ligand binding domain (LBD) is moderately conserved among different receptors. Distinct parts of this domain play essential roles in homodimerization, heterodimerization, and interaction with transcription cofactors affecting transcriptional activity (reviewed in 1, 2, 3). The LBD also contains an activation function 2 (AF-2) motif which essentially exhibits ligand-dependent transactivation function in classical

nuclear hormone receptors. However, several orphan nuclear receptors including constitutively activated receptor (CAR) (4) and estrogen related receptor (ERR) (5) show constitutive activity of AF-2 in the absence of any added ligand.

With the advent of current molecular biotechnology, a large number of nuclear receptors have been cloned from animals ranging from jelly fish to human. Although biological functions of most of the orphans are unknown, a large body of evidence, especially insights gained from gene targeting strategies, indicate very important roles for orphans. Disruption of ERRβ causes severe placental abnormality (6), and disruption of SF-1 in mice causes lack of adrenal glands and gonads with consequent male to female sex reversal of their internal and external genitalia (reviewed in 7). Moreover, early embryonic lethality is associated with the homozygous loss of HNF4 gene in mice, and loss of a single copy of HNF4 in human causes the MODY syndrome (maturity onset diabetes of the young) (8-10).

The TR2 subfamily of nuclear hormone receptors mainly consists of two well characterized receptors named TR2 and TR4. These receptors and their several isoforms have been cloned from mouse, human, and rat (11-15). Moreover, amphibian members of this group of receptors, developmental receptor 1 (DOR1) and developmental receptor 2 (DOR2), have also been cloned from *Ambystoma* and *Xenopus*, respectively

(16, 17), although these amphibian forms are yet to be functionally characterized. The mammalian forms of TR2 are expressed at a high level in adult testis and particularly in the postmeiotic germ cells (11-14). Previous studies have demonstrated that TR2 is a potent repressor of retinoic acid (RA), thyroid hormone (T3) and vitamin D3 mediated transcriptional activity of their cognate receptors via competitive binding to the hormone response elements of DR series (18-25). However, a recent report also demonstrated a role of TR2 as an activator of RARβ2 gene promoter where TR2 in association with CREM activates the gene (26).

In the current study, we report the molecular cloning and characterization of a novel chicken orphan nuclear receptor TRR. TRR encodes a protein of 569 amino acids. Among the adult tissues examined, TRR mRNA is highly expressed in lung, testis, kidney, and small intestine whereas a lower level of expression is evident in skeletal muscle, heart, brain and liver. Expression of TRR mRNA in chick embryo is detected in all the stages examined including developing head and trunk segments. The DNA binding analysis showed significant binding activity of TRR on DR1, DR4 and DR5 sequences, and in consistence with these DNA binding results TRR also forms a DNA-Protein complex with a PBRU described previously as a specific response element for the chicken xenobiotic receptor (CXR) (27, 28). *In vitro* and *in vivo* interaction studies

revealed that TRR forms homodimers. Finally, transient transfection studies confirmed activation of canonical DR1, DR4, and DR5 elements mediated by TRR and the molecular domain responsible for this activity was mapped to the N-terminal A/B domain of this receptor.

MATERIALS AND METHODS

Isolation of TRR cDNA

Chicken liver cDNA library (stratagene) was screened with the DNA binding domain of mouse constitutively activated receptor (CAR) as a probe using low stringency hybridization and washing conditions. Briefly, about $6x10^5$ plaques were plated and immobilized on Hybond-N nylon membranes (Amersham Pharmacia Biotech). The hybridization was carried out at 55^0C in hybridization buffer (6XSSC, 0.5% SDS, and 10X Denhardt's solution without salmon sperm DNA. The membranes were washed 3 times (15 minutes each) at room temperature in 3XSSC, 0.1% SDS after 40 hrs of hybridization and autoradiographed. Finally, positive clones were excised from the phage to the plasmid Bluescript using *in vivo* excision by the Exassist system supplied with the library, and sequenced by the dideoxy sequencing method.

Plasmids

TRR cDNA was excised from the plasmid pBluescript and inserted into pcDNA3 mammalian expression vector at BamH1 and XhoI sites. A polymerase chain reaction (PCR) amplified fragment of the TRR LBD corresponding to amino acids 139-

571 was cloned into EcoRI and XhoI sites of pL202PL and cgatrp (29) for the LexA and B42 fusion expressions in yeast. The mammalian two hybrid DNA constructs were cloned by inserting the same fragment into EcoRI and SalI sites of pCMX-GAL4 and EcoRI and XhoI sites of pcDNA3-VP16 vectors. For the GAL4A/B domain construct, a PCR amplified product corresponding to the N-terminal 74 amino acids of TRR, containing an in-frame stop codon was cloned into EcoRI and SalI sites of a pCMX-Gal4 vector. DR1, 4, and 5 driven luciferase reporter constructs were kind gifts from Dr. Jae Woon Lee.

***In vitro* translation**

TRR cDNA in pBluescript was transcribed and translated *in vitro* by using a coupled Rabbit reticulocyte system (TNT, Promega) in the presence of [^{35}S] methionine (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The translated protein was analyzed on 10% SDS-polyacrylamide gel, and visualized by autoradiography.

Northern blot analysis

30μg of total RNA from the indicated tissues from an adult broiler breeder was isolated and Northern blot analysis was carried out as described previously (30). To

obtain RNA from the various stages of chick embryos, fertilized chick eggs were incubated at 38.5^{0}C in a humidified forced-draft incubator for 75-150 h. At the time of harvest, the embryos were staged according to Hamburger and Hamilton (31). The harvested embryos at the stages between 19-28 were washed several times in phosphate buffered saline (PBS), and quick frozen in dry ice-isopropanol bath and stored at -70^{0}C until use. Total RNA from the indicated stages was extracted and northern blot analysis was performed as described previously (30).

Electrophoretic Mobility Shift Assay

Chicken CXR, chicken TRR and chicken 9-cis-retinoid X receptor gamma (RXRγ) were synthesized *in vitro* using the TNT T7 Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instructions. Probes were labeled and purified over a Biospin 30 Chromatography Column (Biorad). A volume of labeled oligonucleotide corresponding to 100'000 cpm was used for each reaction in 10 mM Tris (pH 8.0), 40 mM KCl, 0.05% NP-40, 6% glycerol, 1 mM dithiothreitol, 0.4 mg/ml BSA, 0.2 μg poly(dI-dC and 2.5 μl of each of the in vitro synthesized proteins as described previously (28). To test for supershifts, 0.5 μl of monoclonal anti-mouse-RXR rabbit antibody (kindly provided by Dr. P. Chambon,

IGBMC, Université Louis Pasteur, Illkirch, France) was added to the reaction mix. In case of competitions unlabeled oligonucleotides at a 50 fold molar excess was used.

Yeast two hybrid assays

Yeast two hybrid interaction studies were performed as previously described (32). Briefly, LexA TRR LBD was co-transformed with B42 vector alone or B42 fused receptor constructs into *Saccharomyces cerevisae* EGY48 containing the β-galactosidase reporter plasmid, 8H18-34, and the transformants were plated on plates containing the appropriate selection markers. The β-galactosidase assay on plates were carried out as described previously (32).

Cell culture and transient transfection

HeLa cells were seeded in 24-well plates 24 hours before transfection in the Dulbeccos Modified Eagle's Medium (Life Technologies) supplemented with 10% fetal bovine serum and transfected with the indicated plasmids using Superfect (Qiagen), according to the manufacturer's instructions. 48 hours after transfection, cells were harvested and luciferase activity was assayed as described previously (30). The luciferase activity was normalized with the β-galactosidase activity.

RESULTS

Identification of TRR cDNA

In order to find new members of orphan nuclear receptor superfamily, a chicken liver cDNA library was screened using a low stringency hybridization approach with the DBD of mouse constitutively active receptor (mCAR) cDNA as a probe. Among the positive clones selected three independent clones represented for a full length TRR. The complete nucleotide and deduced amino acid sequences were determined (fig.1A). The full-length TRR cDNA was found to consist of 2371 nucleotides. The open reading frame (ORF) starting from the first methionine to the first stop codon consisted of 1707 nucleotides and encoded a protein containing 569 amino acids.

The sequence analysis and subsequent blast searches revealed that TRR bears 72% overall amino acid identity with TR2. The DBD showed 95% amino acid identity, whereas the DEF domains including the hinge region and LBD showed a sequence identity of 75%. The hypervariable A/B domain showed 40% amino acid identity (fig 1B). A closer examination of TRR and its mammalian and amphibian relatives revealed that the first 19 amino acids were remarkably conserved in the hypervariable A/B

domain, indicating that these 19 amino acids may play an essential role in the biological function of these receptors (fig. 2A). However, the minimal sequence implied in the activity of TR2, spanning from N-terminal amino acid number from 25 to 30 (26) were not found to be conserved in both TRR and its amphibian relative developmental orphan receptor 1 (DOR1). Moreover, the C-terminal region of the A/B domain containing amino acids 50 to 100, which had been implied for a masking effect on the N-terminal activation sequence of TR2 (26), were not found to exist in TRR (fig.2A). A closer examination of the C-terminal part of the LBD revealed that the heptad repeat region in the helix 10 of the LBD and the putative AF-2 motif of TR2 and TRR were almost identical, indicating that the co-activator binding and the dimerization properties of TRR might be very similar to TR2 (fig.2B). Taken together, these results suggest that TRR might be structurally and functionally different from TR2, although some of its properties like DNA binding specificity and interaction pattern with other proteins might be similar to TR2.

Expression of TRR

In order to determine the expression pattern of TRR in adult tissues, a Northern blot analysis was performed using the full length TRR cDNA as a probe. High

expression of a 2.4 Kb TRR transcript was found in lung, testis, and kidney, and a lesser level of expression was observed in skeletal muscle, small intestine and heart, whereas very little expression was observed in liver and brain (fig. 3A). The tissue distribution of TRR shows a marked difference from its mammalian relatives, mouse, human and rat TR2s, which were shown to be expressed at a high level in testis only (11,12). This particular difference of expression pattern of TRR suggests a possibility that TRR may play a different role in chicken. As demonstrated in Fig. 3B, Northern blot analysis of an embryonic stage blot revealed that TRR gene is expressed in all the stages examined. However, a higher expression of TRR transcript was detected at stage 26 onwards, and the intensity of expression was equal in both stage 28 head and trunk segments indicating that TRR might be involved in overall development of chick embryo. The SDS-PAGE analysis of *in vitro* translated TRR revealed a protein of approximately 68 kDa (Fig.3C) which is similar to the estimated size of TRR from its predicted amino acid sequence.

DNA binding analysis of TRR

To determine the DNA binding pattern of TRR, gel mobility shift assays were performed with the oligonucleotide probes ranging from DR0 to DR6. TRR was found

to form specific high affinity DNA-protein complexes with DR1, DR4 and consensus DR5 only (Fig. 4A). This finding was rather surprising considering that previous reports have demonstrated high affinity binding of TR2 on all the direct repeat elements ranging from DR1 to DR6 including βRARE (18, 20, 24). Moreover, TRR also failed to bind βRARE (data not shown) and therefore βRARE was used as a nonspecific competitor in the competition assays. Taken together, these results demonstrate that TRR shows a considerably different binding pattern despite having a 95% sequence identity in DBD and identical P box and D box motif with TR2. Based on these results, we assume that the sequence identity in the DBD may not be the only criterion for DNA recognition and binding specificity of TRR, and we cannot rule out the possibility that other domains may have definite roles in DNA binding. In consistence with the gel shift results using cannonical direct repeat sequences TRR formed specific complexes with a DR-4 motif located in phenobarbital-responsive enhancer units (PBRU) from the 5'-flanking region of the chicken drug-inducible cytochrome P450 CYP2H1 (Fig. 4B). These DR-4 motifs have been shown to be crucial for mediating drug-induction of these enhancer elements (27, 33). RXR alone does bind to the PBRUs (lane 2) whereas, TRR forms a homodimeric complex with the probes (lane 3) that is not influenced by addition of RXR (lane 4) or RXR and anti-RXR antibody (lane 5). As control, CXR/RXR

heterodimers bind to the more proximal 264-bp PBRU (left blot) and the distal 240-bp PBRU (right blot) as described elsewhere (27,).

TRR forms homodimers

Since TRR shares an identical 9th heptad sequence with all TR2 family members, we attempted to examine the interaction pattern of TRR. As expected, TRR formed homodimeric complex in the yeast two hybrid interaction assay and activated the LexA response element driven β-galactosidase reporter gene in the presence of both LexA-TRR and B42-TRR (Fig. 5A). However, TRR failed to interact with RAR or RXR and co-factors NcoR and SMRT. To confirm the homodimerization activity of TRR *in vivo*, a mammalian two hybrid approach was used. The LBD of TRR was cloned in a CMV promoter driven mammalian expression vector containing GAL4 DBD and also in a vector encoding VP16 activation domain. Transient transfection assays were performed in HeLa cells, using a GAL4-dependent luciferase reporter gene. Upon co-transfection, GAL4-TRR-LBD interacted with VP16-TRR-LBD and thus activated the reporter activity significantly (Fig. 5B). However, GAL4-TRR-LBD alone strongly repressed the basal reporter activity, indicating that TRR behaves as other liganded receptors, which show repressive activity in the absence of ligand. Taken

together, these results clearly demonstrated that TRR exerts its biological effect in the form of a homodimer. This phenomenon is typical in TR2 subfamily of receptors, which are not shown to form heterodimeric complex with RXR despite bearing an identical heptad repeat in the helix 10 with RXR. However, it remains to be determined whether TRR can interact with the known TR2 interacting proteins such as RIP140 and TR4 (34, 35).

TRR shows transcriptional activity

Previous studies demonstrated the biological activity of TR2 to be primarily repressive on both RA induction of DR1 and DR5 elements, and T3 induction of DR4 elements. Previous reports have also shown that the DBD and LBD of TR2 are sufficient for this repressive action (18, 21, 24). To investigate the effect of TRR on the elements it binds, transient transfection studies were carried out in HeLa cells. Interestingly, TRR alone was sufficient for the activation of the elements DR1, DR4, and DR5 ((Fig.6), while it did not show any transcriptional activity on a minimal TK-luc element (data not shown), thus confirming the activation of the DR1, DR4 and DR5 by TRR to be a specific activity which is dependent on DNA binding. These results clearly suggest that TRR is an active orphan nuclear receptor and this receptor

might be a more potent transcriptional activator than its mammalian relatives. However, as demonstrated in Fig. 5B, GAL4-TRR-LBD repressed the basal promoter activity of GAL4TK-Luc, we assumed the LBD and specially the AF-2 may not be the domain in TRR responsible for this transcriptional activity. Since all the TR2 family members show a remarkable identity in the otherwise hypervariable N-terminal region we assumed this region might be important for TRR transcriptional activity. To investigate this possibility a GAL4DBD fused TRR A/B domain representing the amino acids 1 to 74 or GAL4DBD fused TRRLBD were transfected in HeLa cells. Whereas TRRLBD repressed the basal promoter activity, the A/B domain showed a significant transcriptional activity, thereby activating the promoter in a dose-dependent manner (Fig. 7). These results indicate that TRR A/B domain may contain a ligand independent activation function. However, we can not rule out the possibility that a yet unidentified ligand exists for TRR which is needed for the complete transcriptional activity of this orphan receptor.

DISCUSSION

Here, we describe the isolation and characterization of a novel avian orphan nuclear receptor called TRR, which appears to belong to the TR2 subfamily of orphans as it bears highest amino acid identity with TR2. Analysis of the TRR sequence suggests that TRR may not be an orthologue of mammalian TR2. First, the overall identity between TR2 and TRR is only 72%. The A/B domain bears 40% identity, and the ligand binding domain bears 75% identity with TR2 (Fig. 1B), whereas human and chicken COUP-TFIIs bear an overall identity of 93% and a 99% identity with the LBD. Moreover, the difference in expression pattern and DNA binding properties also support the idea that TRR is different from TR2 in its structure and function.

A critical analysis of TRR structure demonstrated many interesting differences of this receptor with its mammalian relative TR2. A closer examination of the hyper-variable A/B domain showed that the first 19 amino acids in TRR were remarkably identical to both TR2 and its amphibian relative DOR1 (Fig. 2A), indicating that this region might play a very important role in the biological functions of this group of receptors. However, the minimal sequence of TR2 implicated in the activation function

of TR2, spanning from N-terminal amino acid 25-30 (26) was not found to exist in TRR or DOR1. Moreover, the amino acids from position 50-100, which had been previously implied to have a masking affect on the transcriptional activity of TR2 (26) were not conserved in TRR. Interestingly, TRR showed a gap of 17 amino acids from amino acid numbered 52, and these amino acids were interestingly found to be conserved in both human and mouse TR2 and DOR1. However, the LBD of TRR showed a striking homology with TR2 in the heptad repeat in the helix 10 (Fig. 2B), indicating that dimerization properties of the two receptors might be identical. Moreover, a critical comparative analysis of the LBD revealed that a conserved basic amino acid residue in the “signature motif”, arginine or lysine in all the nuclear hormone receptors with the exception of NGFIB which contains a glutamic acid instead, was substituted by a hydrophobic residue (leucine) in TR2. In contrast, this basic residue was conserved in both TRR (Fig. 1A), and DOR1. This observation indicates that the leucine residue observed in all the mammalian homologs of TR2 might have originated later in evolution. Based on these structural analyses we suggest that TRR may have originated as a separate lineage in the course of evolution and attained very specialized structural as well as biological parameters and properties. Our hypothesis is also duly supported by the fact that tissue distribution of TRR shows a marked difference from its

mammalian and amphibian relatives.

High expression of an approximately 2.4 kb transcript of TRR was observed in lung, testis, kidney and small intestine (Fig. 3A), whereas TR2 expression is confined to mid gestation embryonic stages and adult testis only (11). The expression pattern clearly suggests that the role of TRR might be much different from TR2. Surprisingly, TRR was found to be expressed in all the embryonic stages examined (Fig. 3B), and the expression seems to increase at about stage 26 onwards when rapid development of alimentary tract and other visceral organs takes place (44), and the intensity of expression remains constant in stage 28 in both head and trunk segments indicating that TRR may play an important role in overall organogenesis. however, detail expression analysis for TRR in a wider range of embryonic stages as well as its localization in different embryonic tissues is required to fully understand the role of TRR in chicken emryonic development, and such studies are currently on going in our laboratory.

DNA binding analysis demonstrated that TRR binds efficiently to DR1, DR4, and DR5. In consistence with the gel mobility shift assay results with cannonical DR series sequences TRR also formed specific homodimeric complex on the DR4 motif of two distinct PBRUs, although the, binding affinity for TRR on these elements was lower

than CXR-RXR heterodimer, but this receptor did not show any specific binding activity to DR0 or DR2 (Fig. 4A and 4B). Moreover, TRR showed little or no binding activity to other members of DR elements such as DR3, DR6 and βRARE (data not shown). Previous studies have demonstrated that TR2 can bind all of the direct repeat elements ranging from DR1 to DR6 in the following order of affinity: DR1 > DR2 > DR5, DR4, DR6 > DR3 (18). This was highly surprising considering the fact that TRR bears 95% amino acid identity with TR-2 in the DBD. Interestingly, a similar phenomenon has also been observed in the case of estrogen receptor (ER) α and β. ERα efficiently binds a consensus steroidogenesis factor 1 response element (SFRE), but ERβ fails to bind it in spite of an almost identical DBD differing by two amino acids only (39). Based on this result, we suggest that DNA recognition and binding specificity of nuclear receptors may be determined not only by the P box of DBD but also by other domains particularly the LBD. Our view is also supported by a previous observation in which substitutive mutation of conserved glutamic acid residues at amino acid numbered 553 and 554 in the LBD of TR2 drastically changed the DNA binding affinity of the receptor (24).

Both *in vitro* and *in vivo* interaction studies with the TRR LBD demonstrate that TRR can form homodimers, and this receptor did not show any interaction with

RXR, which is a common heterodimerization partner of many receptors (Fig. 5A and 5B). Interestingly, members of the TR2 subfamily of receptors are structurally very close to RXR subfamily members and they share an almost identical heptad repeat in the helix 10 region which is believed to be an essential region for dimerization properties of nuclear hormone receptors. Thus it remains an interesting question why TR2 family members do not share similar dimerization properties with RXR. TRR LBD did not show any interaction with RAR or cofactors like NCOR, and SMRT. In fact, a wide array of nuclear hormone receptors including, ER, GR, VDR, T3R, ERR, mCAR, SHP, DAX-1, SF-1 and cofactors like SRC-1 and CBP/P300 were tested for interaction with TRR LBD (data not shown) but we did not observe any interaction of TRR with these proteins. However, it remains to be determined whether TRR can interact with the known TR2 interacting proteins, like TR4 and RIP-140 (34, 35). It also remains to tested whether the A/B domain of TRR can interact with proteins involved in the basal transcriptional machinery or with transcriptional cofactors.

Transient transfection studies revealed that TRR efficiently transactivates DR1, DR4 and DR5 DNA recognition elements ((Fig. 6), whereas, TR-2, was found to be primarily repressive on most of the elements tested, and it was transcriptionally active

only in the context of the specific promoter of RARβ2 (26). This contrast in the behavior of TR2 and TRR indicates that TRR might act as a more potent transcriptional activator of its putative targets than TR-2. This hypothesis also gains support from the fact that TRR could not repress T3 or RA induced activity of DR1, DR4, and DR5 elements (data not shown). Although TRR binds to the 264bp and 240bp CYP2H1 PBRUs (Fig. 4B), we could not observe an effect of TRR on drug induction mediated by these elements. In CV-1 transactivation assays, TRR could neither be activated by prototypical inducers, such as phenobarbital, propylisopropylacetamide, dexamethasone, metyrapone, β-naphtoflavone, clotrimazol or rifampicin nor was any repressing effect on the extent of induction mediated by chicken xenosensing receptor CXR found (data not shown). These findings are corroborated further by experiments in the drug-responsive chicken hepatoma cell line LMH, where transfection of TRR did not influence drug response as measured by reporter gene (data not shown). Therefore, binding of the CXR/RXR heterodimer is dominant over TRR binding which is also evident from gel mobility shift asaays performed with two different chicken PBRUs as probes (Fig. 4B). Nevertheless, further investigations will have to be done to examine the role of TRR in hepatic drug-induction of cytochromes P450.

Interestingly, the LBD of TRR showed a repressive activity, which was

transferable to a heterologus protein (GAL4DBD), indicating either the AF-2 region is silent or a specific ligand is needed for its activity. However, a potent ligand independent activation function was found in the N-terminal 72 amino acids which could confer its transcriptional activity to a GAL4DBD (Fig. 7), indicating that the silent AF-2 may not be sufficient to mask this activity. In contrast to TR2, in which the activation function was found to exist in the N-terminal amino acids 10-30, and particularly the amino acids 25-30 which were found to be the most important region for the activation function of TR2, those amino acids were not conserved in TRR. Moreover, the amino acids 50-138 in TR2, which were described to have a masking effect on the AF-1 activity, were also not conserved in TRR. However, it was highly interesting to note that the amino acids 1-19 were remarkably conserved in all the TR-2 family members indicating that this region might play an important role in the transcriptional activity. We also assume that it’s perhaps due to the absence of any masking region in the A/B domain of TRR, this receptor showed significant transcriptional activity on all its recognition elements. This view is in consistence with a previous report (26) where the full A/B domain of TR-2 did not show any transcriptional activity. However, it remains important to map the activation function in TRR as well as finding the specific transcriptional co-factors, which might interact with this region, and such studies are

currently being pursued in our laboratory. Finally, to identify the possible ligand for TRR, several potential activators for nuclear receptors were tested using either full length TRR on DR1-Luc reporter element or GAL4TRR LBD using a GAS-Luc reporter construct, but no significant activation of TRR was observed, indicating that a specific and yet unidentified ligand might be required for the AF-2 domain of TRR to be transcriptionally active.

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Acknowledgements

We thank Dr. Pierre Chambon for anti-mouse RXR antibody, and Dr. Jae Woon Lee for the DR1, 4, and 5 driven luciferase reporters and critical reading of this manuscript.

FIGURE LEGENDS

Fig. 1. Structure and sequence of TRR. (A) Nucleotide and the deduced amino acid sequence of TRR clone. The 5' and 3' non coding regions are shown, and the complete coding region of TRR is shown with its amino acid sequence. The DNA binding domain is underlined and signature motif in LBD is indicated with shaded box. (B) Schematic representation of TRR protein and comparison with other members of nuclear receptor superfamily. The positions of putative initiation and terminations are indicated as a number, the A/B domain, C domain and DEF regions are indicated. The percentage of amino acid identity in each relevant region between TRR and other orphans was determined using domain by domain allignment with Macvector software from Macintosh.

Fig. 2. Sturctural comparison between TRR and other members of the nuclear hormone receptor superfamily. (A) Comparison of the N-terminal sequence of TRR, mTR2, hTR2, and DOR-1. The N-terminal sequence of TRR orphan nuclear receptor and other close relatives were aligned, the conserved first 19 amino acids are marked by an overhead bar. Dashes (---) represent the gaps included for the appropriate

alignment. Asteriks (*) represent identical amino acids and the dots (.) represent amino acids similar in chemical properties. (B) A summary of the C-terminal amino acid sequences of TRR, hTR2, mTR4 and cRXRγ. The C-terminal sequence of TRR and other close relatives were aligned, the conserved 9th heptad repeat and the putative AF2 are in bold, and are marked with overhead bar. The conserved glutamic acid residues are shaded in gray, and the helix 10, 11, and 12 regions are underlined. Dashes (--) represent the gaps included for the appropriate alignment.

Fig 3. Tissue distribution and expression of TRR. (A) Tissue distribution of TRR. A Northern blot was prepared by using approximately 30 μg total RNA extracted from the indicated tissues of an adult broiler breeder, and hybridized with full length TRR cDNA. The arrows indicate the positions of 28S and 18S ribosomal RNA. The lower panel represents 28S ribosomal RNA as a loading control. (B) An embryo stage blot was prepared using approximately 30μg of total RNA prepared from indicated embryonic stages (31), and hybridized with a full length TRR cDNA probe. The lower panel represents 28S ribosomal RNA as a loading control. (C) SDS-PAGE analysis of *in vitro* translated TRR. Full length TRR in pBluescript was *in vitro* translated and loaded on a 12% polyacrylamide denaturing gel. The numbers on the left denote the

molecular weight marker in kDa, and the arrow on the right denotes the position of the protein band.

Fig 4. Binding analysis of TRR. (A) A gel shift analysis was performed using *in vitro* translated TRR protein with end labeled direct repeat DNA sequences spaced by 0, 1, 2, 4, and 5 base pairs (DR0, DR1, DR2, DR4, DR5). Lanes indicated as L; unprogrammed lysate only, P; in vitro synthesized TRR only, SC; specific competition using 50 fold molar excess of cold probe, NC; nonspecific competition using 50 fold molar excess of unlabeled nonspecific DNA. The arrow on the left hand side indicates specific protein-DNA complex, and ** indicates nonspecific complexes.. **(B)** Radiolabeled 264-bp PBRU (left blot) (27) and 240-bp PBRU (right blot) (28) were incubated with in vitro transcribed/translated chicken RXRgamma (lanes 2, 4, 5 and 6), TRR (lanes 3, 4 and 5), chicken CXR (lane 6), anti-RXR antibody (lane 5) or mock programmed lysate (lane 1). The black arrows depict complexes of homodimerized TRR and the probe whereas the gray arrows indicate complexes of CXR/RXR heterodimers with the probe resulting in shifts.

Figure 5. TRR forms a homodimer both *in vitro* and *in vivo.* (A) TRR forms homodimer *in vitro*. A yeast strain EGY418 which contains an integrated β galactosidase reporter gene controlled by LexA binding sites was transformed with PEG202-TRR LBD encoding LexADBD/TRR LBD, and PEGJ4-5-RAR, RXR, NcoR, SMRT or TRR LBD encoding B42AD/RAR, RXR, NcoR, SMRT or TRRLBD. The transformants were selected on plates containing appropriate markers and assayed for β-galactosidase activity. LexAN and B42N represent respective empty vectors used as a negative control. **(B)** TRR forms homodimer in vivo. 0.2 μg of pcDNA3 GAL4TRR encoding GAL4DBD/TRR and 0.2 μg of pcDNA3 VP16TRR encoding VP16AD/TRR were transiently transfected to HeLa cells, with 0.4 μg of GAL4-TK LUC. GAL4 N and VP16 N are empty vectors used as negative controls. The luciferase activity was measured and normalized against β-galactosidase activity. One representative result is shown, similar results were obtained with at least three independent experiments.

Fig.6 TRR specifically activates canonical DR1, DR4 and DR5 elements. HeLa cells were co-transfected with canonical DR1 **(A)**, DR4 **(B)**, and DR5 **(C)** element driven luciferase reporter constructs with 0 (mock)100, 200 or 500ng of pcDNA3 TRR as

indicated in the figure. The luciferase activity was measured and normalized against the β-galactosidase activity. The result shown is the mean of three independent experiments and the error bars indicate the standard deviation. Similar results were also obtained from co-transfection experiments in CV-1 cells.

Fig.7 A/B domain of TRR shows constitutive transcriptional activity. HeLa cells were co-transfected with a 5 copy GAS-luc promoter and indicated doses of GAL4-TRRLBD or GAL4-TRRA/B as indicated in the figure. The luciferase activity was measured and normalized against the β-galactosidase activity. One representative result is shown, similar results were obtained with at least three independent experiments, and the result was also reproducible in CV-1cells.

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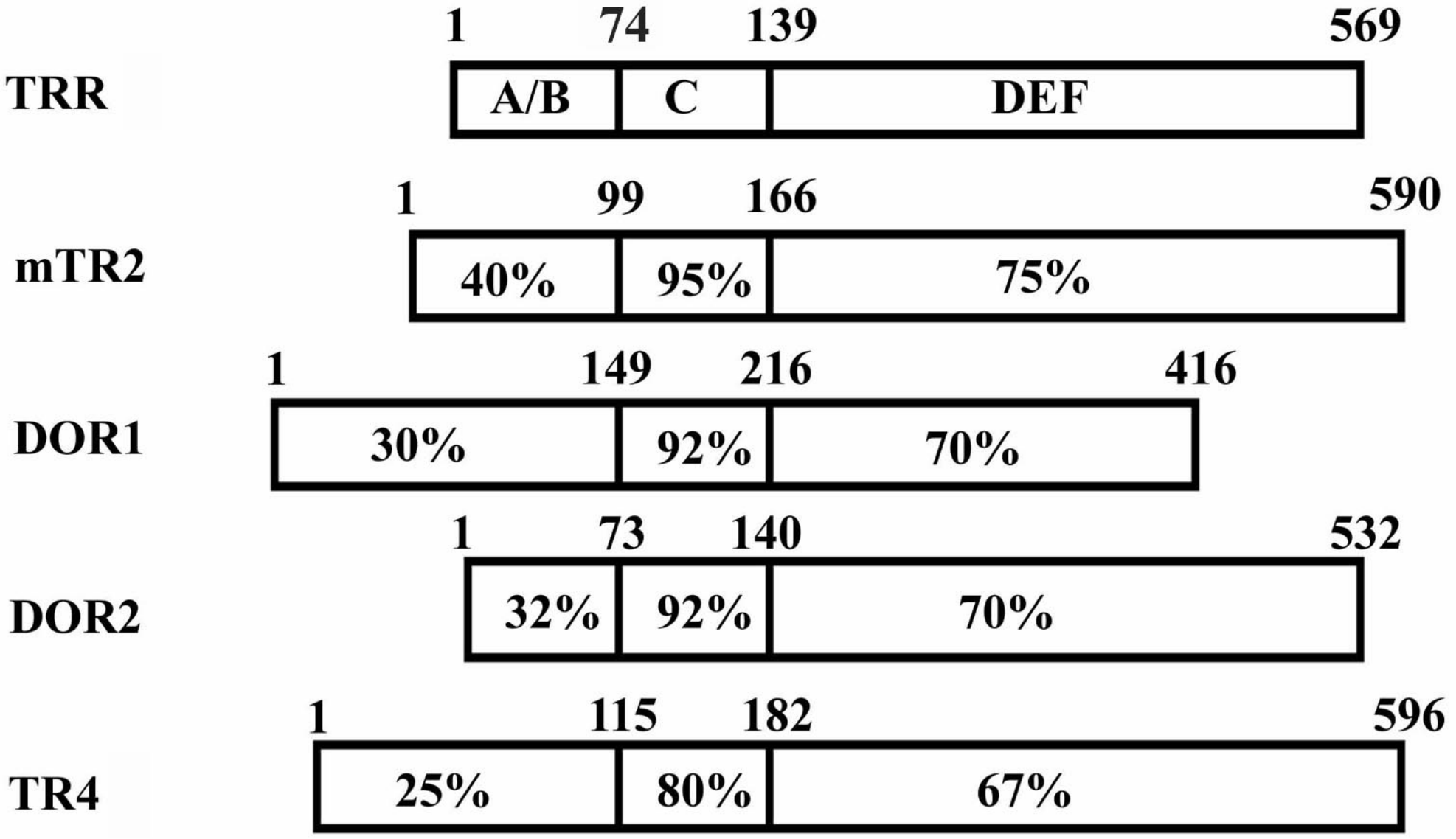
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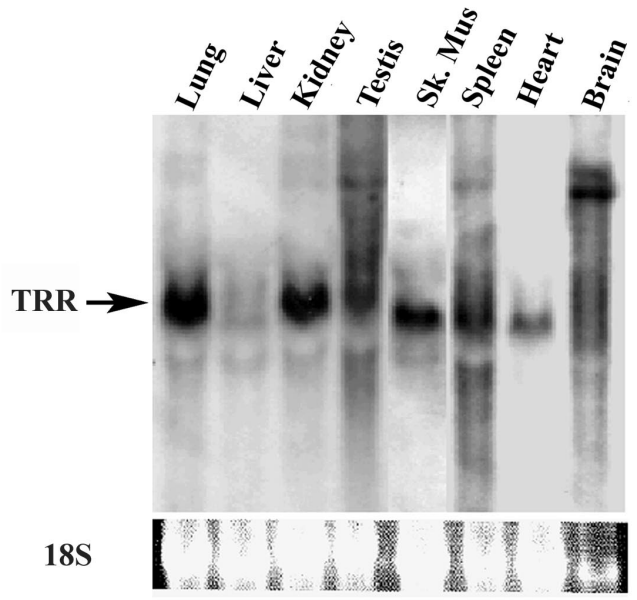
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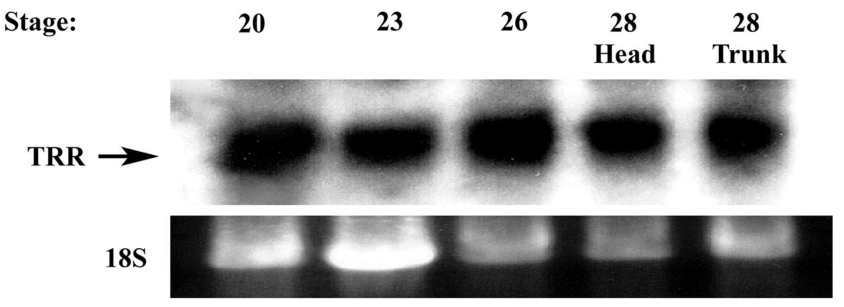
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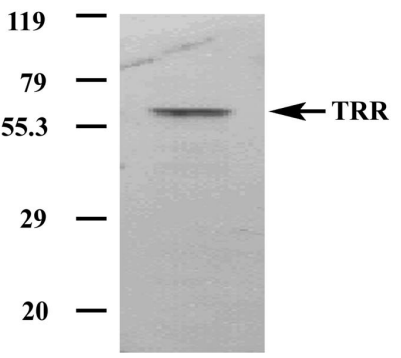
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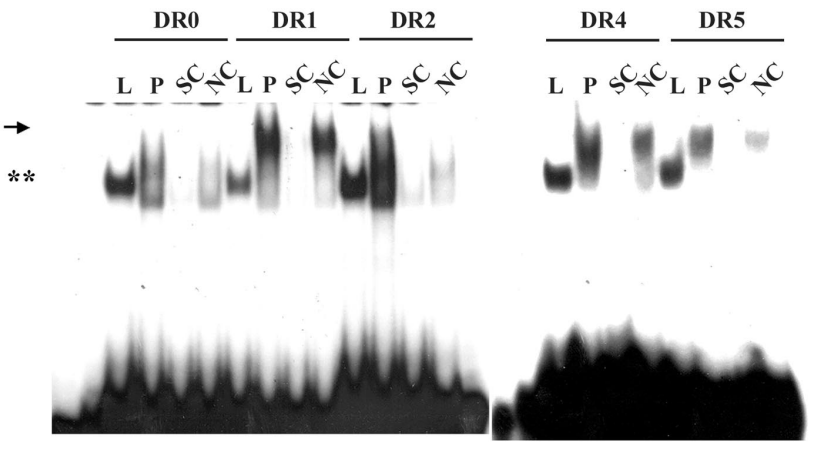
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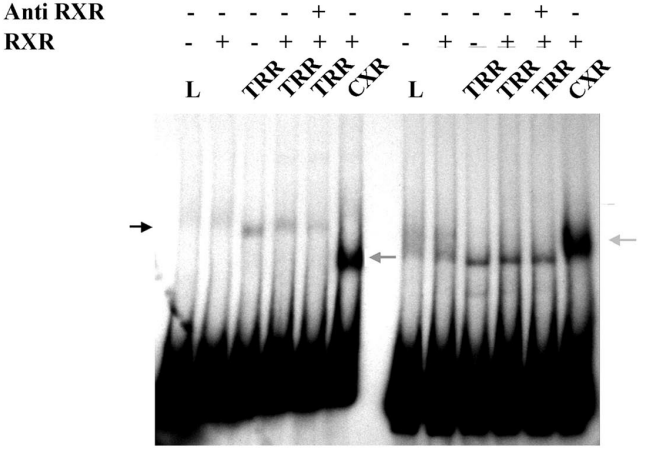
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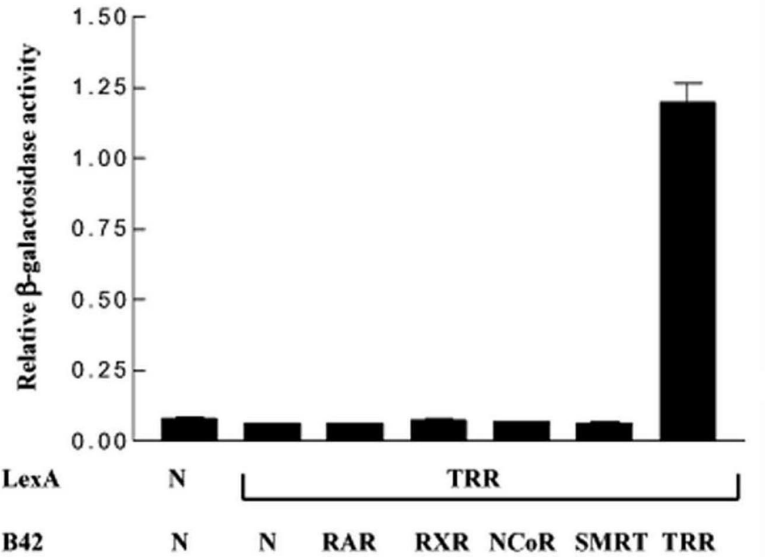
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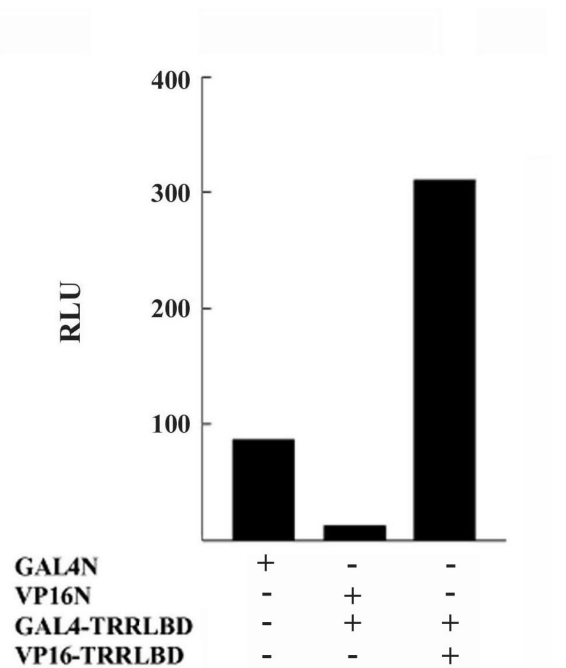
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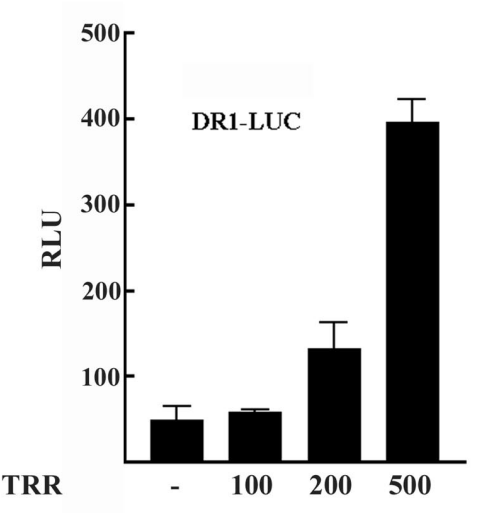
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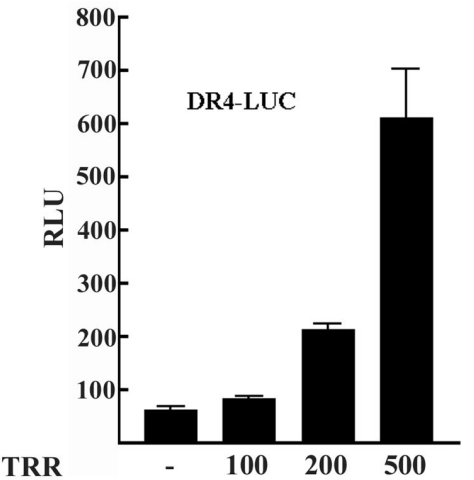
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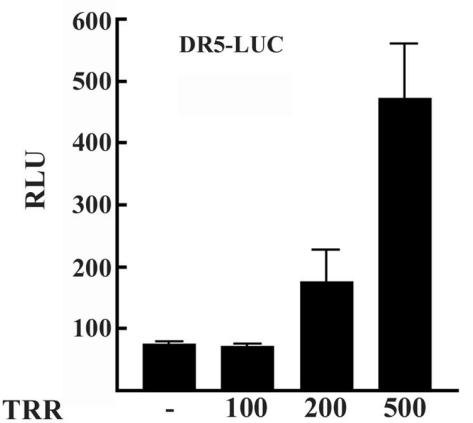
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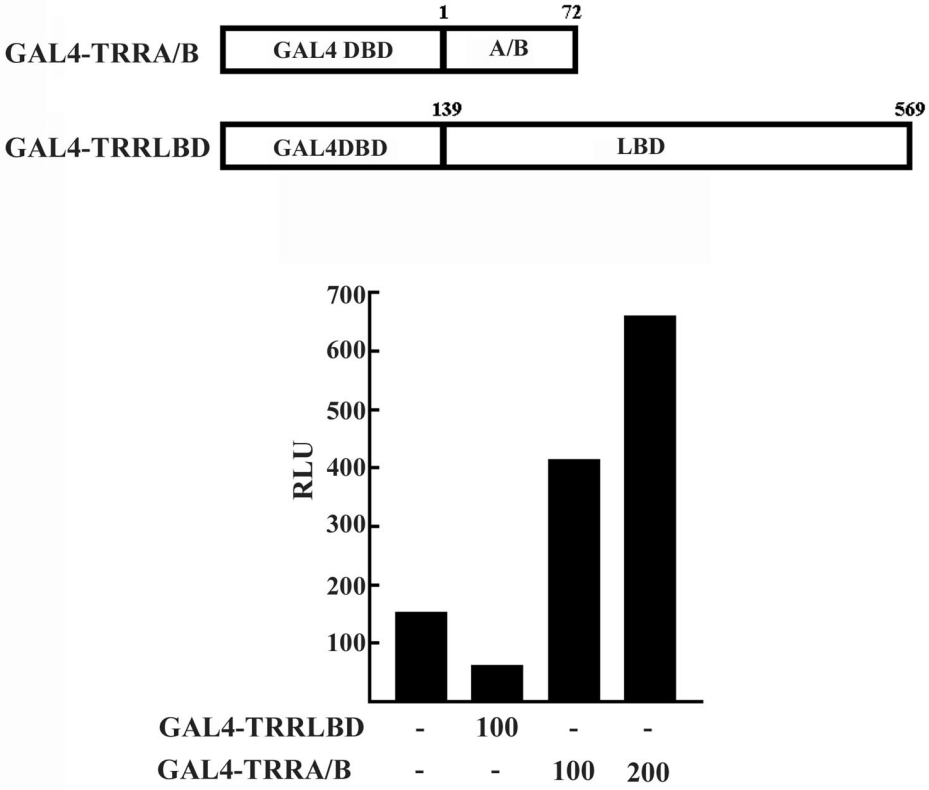
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GAL4-TRRA/B
GAL4 DBD
A/B
139
569
GAL4-TRRLBD
GAL4DBD
LBD
700
600
500
400
300
200
100
RLU
GAL4-TRRLBD - 100 - -
GAL4-TRRA/B - - 100 200