The mouse orphan nuclear receptor TR2-11 functions as a repressor for reporter genes containing a direct repeat-5 or direct repeat-4 hormone response element. The functional domains responsible for its suppressive activity are defined, including the DNA-binding domain and the ligand-binding domain. The C-terminal 50 amino acid residues can be deleted without compromising its suppressive activity, whereas a deletion for 40 amino acids completely abolishes the suppressive activity and receptor dimerization, and reduces the DNA-binding affinity. Point mutation at three conserved leucine residues located on the predicted dimer interface abolishes the suppressive activity, receptor dimerization and its DNA binding property. However, mutation at two consecutive glutamate residues located within the hinge between the last two helices of the ligand-binding domain (helix 10 and helix 11 according to the human retinoid receptor X α structure) drastically reduces its DNA-binding affinity and abrogates the suppressive activity without compromising its ability to dimerize, indicating that receptor dimerization property can be functionally uncoupled from its suppressive activity. A transferable, active silencing activity is encoded within the DEF segment of the receptor molecule, as evidenced by the suppression of a GAL4 reporter by a chimeric protein containing the DNA-binding domain of GAL4 and the DEF segment of TR2-11. Moreover, the C-terminal 49 amino acid sequence is required for this trans-suppressive activity. It is suggested that TR2-11 functions as a repressor, mediated by mechanisms requiring high affinity DNA binding, receptor dimerization, and active silencing.

Nuclear receptors constitute a large family of transcription factors that play key roles in gene regulation. Many of these receptors are transcription regulators that modulate target gene expression by binding to specific DNA sequences in its promoter region (1–6). These include the receptors for glucocorticoid, estrogen, progesterone, vitamin D, thyroid hormone, and retinoic acid, as well as a large number of orphan members that have no known ligands. The nuclear receptors share a common modular structure consisting of a variable N terminus, a DNA-binding domain (DBD) in the middle of the receptor molecule, and a C-terminal ligand-binding domain (LBD) (1, 2). The LBD determines the ligand specificity by formation of a highly specific binding pocket and mediates receptor dimerization by interacting at the dimerization interface located on the LBD surface. In their apo-forms, nuclear receptors function as repressors by interacting with corepressors, such as the nuclear receptor corepressor (N-CoR) (7) and silencing mediator for retinoid and thyroid hormone receptors (SMRT) (8). These corepressors interact with the apo-receptors at their hinge regions between the DBD and LBD. Ligand binding induces a conformational change of the receptor, resulting in the release of the corepressor and recruiting of the co-activator, which binds to the activation function-2 (AF-2) domain located at the C terminus of the receptor molecule (9–14). Direct interaction with the components of the basal transcription machinery such as transcription factor IIB and TATA box-binding protein (TBP) has also been implicated in the ligand-independent silencing activity of some of these receptors (15, 16).

The mouse TR2-11 is a member of the orphan receptors and is expressed mainly in the developing germ cells in the testis (17, 18). Several mRNA isoforms have been identified (19–20), including one that encodes the full-length receptor and an alternatively spliced isoform that encodes a truncated receptor deleted in the entire LBD (19). Our previous studies have shown that the full-length receptor strongly suppresses reporters containing either a direct repeat-5 (DR5) (18, 19) derived from the human RARβ gene promoter (21), or a DR4 type response element (22) derived from the mouse cellular retinoic acid-binding protein-I gene promoter (23). In contrast, the truncated receptor exerts no consistent biological activities in similar transient transfection experiments, despite the presence of an intact DBD. By comparing the primary structures of the nuclear receptors, the full-length TR2-11 receptor is much larger than most other nuclear receptors due to the extraordinary large DEF segment of its coding region (approximately 435 amino acid residues for TR2-11, as compared with approximately 200–300 amino acids for most other receptors) (24). In order to understand how TR2-11 suppresses gene expression as demonstrated by many previous studies (18, 19, 22, 25, 26), we have set up to dissect the functional domains responsible for its biological activity and to determine whether TR2-11 employs any unique pathway or adopts similar mechanisms employed by other receptors for a strongly suppressive activity.

In this study, the molecular mechanisms underlying the suppressive activity of TR2-11 were dissected in the estab-
lished DR5 reporter system (18). In this system, it has been shown that RA induction of the reporter was dramatically suppressed by the full-length TR-11 receptor, but not by the truncated variant. Here, we first examined the mutant receptors made by deletion and point mutagenesis, in their dimerizing ability and DNA binding property, and compared these properties to their suppressive activities in the DR5 reporter system. We then examined the role of the predicted ninth heptad repeat within the LBD in receptor dimerization, DNA binding, and suppressive activity. We further identified one conserved glutamate residue located between helices 10 and 11, which was critical for the suppressive activity and DNA binding affinity, but not for receptor dimerization. We went on to determine whether its suppressive activity is transferable by using the GAL4 fusion protein system. Finally, we explored the possibility of its interaction with the common corepressor N-CoR as well as heterodimerization with RAR/RXR family.

**MATERIALS AND METHODS**

**Plasmid Constructs**—The reporter, RARE-tk-luc, containing a DR5 was made as described previously (18). The RAR-luc reporter was constructed by fusion of a promoter fragment between positions −99 and −11 of the hRARβ gene (27) into the Mlu I/Xhol sites in front of a promoter-less luciferase reporter, pGL3-Basic vector (Promega, Madison, WI). The cytomegalovirus vector containing the full-length RAR, as well as the deletion constructs, was ligated using the EcoRI/XhoI (590 amino acid residues) for mammalian expression was as described previously (22). The amino acid numbering system was according to our previous study (18). To construct the C-terminal deletions of TR-11, fragments deleted in various sizes from the C terminus were generated by polymerase chain reactions (PCR), flanked by HindIII and XbaI sites and used to replace the HindIII/XbaI fragment of the wild type expression vector. TR2ΔC49, TR2ΔC40, TR2ΔC30, TR2ΔC20, and TR2ΔC10 were each deleted for 49, 40, 30, 20, and 10 amino acids, respectively, from the C terminus. TR2ΔN1 was deleted in the N-terminal amino acid residues 1–99 (the N-terminal variable region), and TR2N2 was deleted in the N-terminal 166 amino acids (the N-terminal variable region and the DBD). Point mutations were generated using a two-step PCR protocol (28). The mutated fragment flanked by HindIII and XbaI sites were used to replace the HindIII/XbaI fragment in the wild type vector. The LLL537–539-YPP mutant was mutated at the consecutive three leucine residues (positions 537–539) by replacing the wild type sequence TTACTTCTC with a mutant sequence TTACTTGTC.

**Transcription/translation reticulocyte lysate system** (TNT system, Promega) was used for in vitro translation of the protein produced in bacteria, GST-TR2 or GST alone, was bound to a glutathione-agarose column and incubated with 32P-labeled RNA. The binding buffer contains 20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 5 mg/ml bovine serum albumin, 10% glycerol, and 10% protease inhibitor mixture. Incubation was conducted for 60 min at 4°C. The bound protein was removed by five washes with the binding buffer without bovine serum albumin and protease inhibitors. The specifically bound protein was eluted with 50 mM reduced glutathione in 50 mM Tris, pH 8.0, and resolved by SDS-polyacrylamide gel electrophoresis.

RESULTS

**Dissection of Functional Domains for the Suppressive Activity of TR-11**—In our previous studies, we have demonstrated that TR-11 exerted a strongly suppressive activity on the reporters containing either a DR5 type RARE (from the human RARβ promoter) (18) or a DR4 type hormone response element (from the mouse CRABP-I promoter) (22). In this study, we have utilized two reporter systems to dissect the mechanisms of the suppressive activity of TR-11. The first was a DR5 artificial reporter (RARE-tk-luc) that had been widely used to study RA induction mediated by DR5 element. In order to confirm the fidelity of responses observed in this artificial reporter, we also included a natural promoter from which this DR5 was derived, the RARβ promoter, a well established gene directly responding to RA induction (27). To first define the functional domains of TR-11 that were required for its suppressive activity, we began by systematic deletions from the N-terminal 49 amino acids, and the amino acid residue 518–590, respectively.

The **Yeast Two-Hybrid Interaction Assay**—Yeast strain YRG-2 containing a laZ reporter controlled by three copies of GAL4 binding sites, the bait plasmid expressing the yeast GAL4 DNA-binding domain and the prey plasmid expressing the yeast GAL4 (pAD) were purchased from Stratagene (La Jolla, CA). A series of bait and prey constructs were made by fusing various fragments of the LBD of TR-11 into the pBD and the pAD vectors. The fragments were synthesized by PCR or retrieved from the existing cDNA vectors. The mRARα prey and bait each contains the full-length receptor. The mRARβ bait and prey each contains amino acids 148–410 (29). The N-CoR prey contains amino acids 1845–2455 (7 fused to the PAD vector. The TR-11 baits include the full-length (TR2), the DEF segment (TR2N2), the DEF deleted in the C-terminal 49 amino acids (TR2ΔC24C49), and the two point mutants (TR2N2-LLL/YPP and TR2N2-EE/RG). The TR-11 preys include the DEF segment (TR2N2), a smaller DEF segment (amino acids 238–590) deleted at its N-terminal portion, TR2ΔN2, as well as the four C-terminal deletions of the DEF segment (TR2ΔN2C10, TR2ΔN2C20, TR2ΔN2C30, TR2ΔN2C40). The positive controls p53 and pSV40 were purchased from Stratagene. The yeast culture, transformation procedures, and determination of lacZ activity were as described previously (22).

**Pull-down Assay**—The full-length TR-11 cDNA was cloned into the pGEX-2T vector (Amersham Pharmacia Biotech) for the production of GST fusion protein. For **in vitro** interactions, a total of 5 μg of fusion protein produced in bacteria, GST-TR2 or GST alone, was bound to a glutathione-agarose column and incubated with 32P-labeled TR-11 protein made in TNT reactions. The binding buffer contains 20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 5 mg/ml bovine serum albumin, 10% glycerol, and a protease inhibitor mixture. Incubation was conducted for 60 min at 4°C. The unbound protein was removed by five washes with the binding buffer without bovine serum albumin and protease inhibitors. The specifically bound protein was eluted with 50 mM reduced glutathione in 50 mM Tris, pH 8.0, and resolved by SDS-polyacrylamide gel electrophoresis.
and C termini of the wild type receptor, and followed by making specific point mutations in the LBD of TR2-11. Fig. 1A shows the maps of two N-terminal deletions, five C-terminal deletions, and four point mutations used in these studies. The suppressive activity of each receptor on RA induction of the RARE-tk-luc and the RAR-luc reporters was assessed in transient transfection assays and the results are shown in Fig. 1 (B and C, respectively). As compared with the transfection with a control expression vector (lane CMV), expression of the wild type TR2-11 (TR2-11-f) or three small C-terminal deletions (lanes TR2ΔC10, TR2ΔC20, TR2ΔC30, and data not shown), resulted in 50–60% inhibition of RA induction on RARE-tk-luc activity and 75–80% inhibition on RAR-luc activity. In contrast, deletion of 49 amino acids from the C terminus (TR2ΔC49) completely abolished the suppressive effect of TR2-11 on both reporters. Interestingly, the 40-amino acid

![Fig. 1. Inhibition of RA induction of the RARE-tk-luciferase reporter by TR2-11, its C- and N-terminal deletion, and point mutants. A, schematic representation of the TR2-11 and deletion constructs used in this experiment. Numbers on the right represent the last amino acid of each construct. TR2-11-f represents the wild type protein, while TR2ΔC denotes the C-terminal deletion followed by the numbers of amino acid deleted, and TR2ΔN1 and TR2ΔN2 represent the N-terminal truncation deleted in the sequence 1–99 and 1–166, respectively. LLL(537-539)YPP construct represents a point mutant whose amino acids at position 537 to 539 (*) has been changed from three contiguous leucine to tyrosine, proline, and proline, respectively. EE(553,554)XX represents point mutants whose amino acid residue 533 and 554 (**) have been mutated from double glutamate to three different expression vectors, designated as EE(553,554)RE, EE(553,554)EG, and EE(553,554)RG. B and C, the folds of RA induction of the RARE-tk-luciferase and RAR-luc reporters in COS-1 cells. COS-1 cells were co-transfected with 0.3 μg of the reporter, 100 ng of the TR2-11 expression vectors, and 50 ng of the internal control (SV40-lacZ) for 20–24 h. LacZ and luciferase activities were determined at 40–44 h to obtained the specific activity. The -fold of RA induction was determined by comparing the specific activity in the presence of RA to that without RA addition. More than three independent experiments were performed. D, localization of GFP fusion of TR2-11 proteins expressed in COS-1 cells. Wild type, deletion mutants, as well as point mutants were tagged with GFP expression vector at the N terminus of the proteins. a, the full-length TR2-11-f nuclear localization pattern. b and c, examples of the deletion and point mutant localization patterns, respectively. d, exclusive cytosolic localization of the TR2-ΔN2 GFP fusion protein.
deletion (TR2 Δ C40) only partially abolished the receptor function in the RAR-luc reporter (Fig. 1C), yet it affected the receptor function in the RARE tk-luc reporter more dramatically (Fig. 1B). As expected, further truncations (94 amino acids or larger) also resulted in the complete loss of the suppressive activity (data not shown). The N-terminal deletion mutant which retained the intact DBD remained suppressive (lane TR2 Δ N1), whereas a further deletion into the DBD completely abolished the suppressive activity (lane TR2 Δ N2). Collectively, these data demonstrate that both the N-terminal variable region and the C-terminal 30 amino acid sequence are not required for the suppressive activity of TR2-11 on either reporter, the region between the 30th and the 40th amino acid from the C terminus exerts slightly different effects between the two reporter systems, whereas the DBD and the LBD are important for the suppressive biological activity in both reporter systems.

Amino acid comparison of TR2-11 and other receptors revealed a striking sequence homology in the region containing the heptad repeats 7–9, which corresponded to helices 9–11 according to the x-ray structure data (30). Within this region, a contiguous LLL sequence located in the ninth heptad repeat is most highly conserved. The LLL sequence was mutated into YPP, designated as LLL(537–539)YPP. As shown in Fig. 1 (B and C), the mutated receptor failed to exert the suppressive activity in both reporter systems. This result demonstrates a critical function of these conserved leucine residues in the suppressive activity of TR2-11.

The observation that deletion of 40 amino acids, but not 30 amino acids, from the C terminus of TR2-11 resulted in a loss of the suppressive activity suggested an important role of the 10 amino acids (between the 30th and the 40th residues from the C terminus) in this receptor activity. Sequence comparison among various nuclear receptors revealed that this 10-amino acid sequence constituted the hinge region between helices 10 and 11, as well as the N-terminal portion of helix 11. Most interestingly, a glutamate residue (Glu-553) in the hinge region was conserved among TR2-11, RXRa, RARα, and RARγ. We then asked whether the hinge region between helices 10 and 11 was important for the suppressive activity. The two consecutive glutamate residues were first mutated to arginine and glycine, designated as EE(553–554)RE and EE(553–554)EG, and tested in transient transfection experiments. As shown in Fig. 1 (B and C), this mutation completely abolished the suppressive activity in both reporter systems. To determine which glutamate residue was important, single mutation was generated, designated as EE(553–554)RE (mutated at Glu-553) and EE(553–554)EG (mutated at Glu-554). We found that mutation at Glu-553 (EE/RE), but not Glu-554 (EE/EG), abolished the suppressive activity. Therefore, it is concluded that the conserved glutamate residue (Glu-553) plays an important role in TR2-11-mediated suppression.

The failure of the mutant receptors to exert an effect on these reporters raised a concern about the possibility of altered mutant protein stability or defect in protein trafficking; we then examined whether these mutant receptors were made inside the cells and properly transported into the nuclei, by using a GFP-tag strategy as shown in Fig. 1D. The wild type receptor tagged with GFP showed a distinct nuclear localization pattern (Fig 1D, a), which was also observed for the receptors of C-terminal deletions (b) and point mutations (c). In contrast, the receptor deleted in the N terminus and the DBD, ΔN2, which was deleted in the putative nuclear localization signal, exhibited a completely cytosolic distribution (d). The N-terminal deletion (ΔN1) also showed a nuclear retention pattern identical to the wild type pattern (data not shown). In addition, protein stability of all these mutant proteins remained relatively constant, as evidenced by the fact that all the nuclear fluorescent signal could be followed for the same duration. We also tested the biological activity of the receptors tagged with GFP in the reporter systems, and a similar pattern of biological activity as that shown in Fig. 1 (B and C) was observed for these GFP-tagged receptors (data not shown). From these results, it is concluded that all the mutant receptors, except ΔN2,
confirm whether dimerization of TR2-11 occurred and if dimerization was also required for the suppressive activity of TR2-11. The ability of TR2-11 and its deletion mutants to form homodimers was examined in the yeast two-hybrid interaction assay. Various portions of TR2-11 were cloned in the yeast expression vectors and tested by co-transfection of the bait and the prey vectors into the yeast. As shown in Fig. 2A, receptors containing the DEF segment remained capable of dimerization (column 2), whereas deleting the C-terminal 49 amino acids rendered receptors unable to dimerize (column 3). Moreover, the interaction between the wild type receptors (column 4) is much stronger than the positive control, between p53 and SV40 large T-antigen (column 1), suggesting a rather strong interaction between the dimer partners. To further dissect the sequence required for this interaction, small deletions from the C terminus were cloned into the prey vector and tested in similar interaction assays as shown in Fig. 2B. In consistence with the results of their suppressive activities (Fig. 1, B and C), receptors deleted for up to 30 amino acids from the C terminus (columns 3–5) remained capable of interacting with the wild type receptor, while further deletion to 40 amino acids (column 6) drastically affected the interaction between the dimer partners. Therefore, it is concluded that deletion from the C-terminal 40 amino acids of TR2-11 abolishes both receptor dimerization property and its ability to suppress target gene expression.

In transfection experiments as shown in Fig. 1, the LLL(537–539)YPP and the EE(553,554)RG mutants completely lost their suppressive activities. We then examined their ability to dimerize in the two-hybrid interaction test. As shown in columns 7 and 8 of Fig. 2, the LLL(537–539)YPP failed to interact with wild type receptor (column 7). Unexpectedly, EE(553,554)RG mutant remained capable of interacting with the wild type receptor (column 8) and the strength of interaction was comparable to that of the wild type receptor (column 2). Collectively, these data indicate that while efficient dimerization is positively correlated with the suppressive activity of TR2-11 (as evidenced by the results of C-terminal deletion and the leucine mutation), other mechanisms mediated by the conserved glutamate residues are also important for the suppressive activity.

To determine whether TR2-11 could form homodimers in a DNA binding-independent manner, pull-down assays were conducted. TR2-11 was tagged with the GST at the N terminus and applied to a glutathione-agarose column. The wild type TR2-11 protein was labeled with [35S]methionine and analyzed by affinity pull-down assay with the indicated GST fusion proteins bound to the glutathione-agarose columns. Lane 1 shows the input control, lane 2 shows the GST alone, and lane 3 shows the positive interaction.

which is deleted in both the N terminus and the DBD, are made, folded properly and transported into the nuclei.

**TR2-11 Receptor Dimerization**—Dimerization is essential for the function of most nuclear receptors. Two dimerization interfaces have been defined, one within the second zinc finger of the DBD and the other within the LBD (31). We first would like to confirm whether dimerization of TR2-11 occurred in vivo and if dimerization was also required for the suppressive activity of TR2-11. The ability of TR2-11 and its deletion mutants to form homodimers was examined in the yeast two-hybrid interaction assay. Various portions of TR2-11 were cloned in the yeast expression vectors and tested by co-transfection of the bait and the prey vectors into the yeast.

**DNA Binding Property of TR2-11**—To ask whether the mutant receptors that lost their dimerization property and suppressive activity, such as receptors deleted at the C terminus or mutated at leucine or glutamate residues, behaved differently in the DNA binding property, we set up the gel retardation experiments using 32P-labeled DR5 fragments as the probes. As shown in Fig. 4A, the wild type receptor (lanes 2–7) was able to shift the DR5 probes to a distinct position labeled with an arrow at the left. This band was competed out approximately 50% by unlabeled fragments at 50-fold excess (lane 7) and supershifted by a specific TR2-11 antibody (lane 3) (22) to a position labeled with a double arrow. The LLL/YPP mutant completely lost its DNA binding property (lane 16), whereas the EE/RG (lanes 12–15) or the TR2ΔC40 (lanes 8–11) mutant remained capable of binding DNA, although at a much lower affinity as demonstrated by almost complete competition by a 50-fold excess of the unlabeled fragments (lanes 15 and 11, respectively). In order to confirm the negative result of the LLL(537–539)YPP mutant, we compared the TNT products for the wild type, EE(553,554)RG, and LLL(537–539)YPP mutants by including [35S]methionine in the TNT reactions as shown in
The specific protein products of these TNT reactions were comparable, indicating that the wild type, LLL(537–539)YPP, and EE(553,554)RG proteins were made equally efficiently. We also found that the specifically shifted band could not be competed by the unlabeled DNA fragments containing either one of the two half-sites and that probes containing one half-site was not shifted by TR2-11 (data not shown), suggesting no binding by TR2-11 monomers. It is apparent that deletion or mutation from the C terminus of TR2-11 drastically affects its DNA-binding affinity.

**Fig. 5.** Binding affinity of TR2-11 to the DR5 sequence. **A**, binding of the *in vitro* expressed TR2-11 and EE(553,554)RG mutant to the indicated amount of unlabeled fragments in gel retardation assay. Constant amounts of proteins were incubated with various amount of the probes as indicated. Lanes 1–5, wild type TR2-11; lanes 6–10, mutant. **B**, Scatchard plot analysis. The ratios between the intensity of the specific DNA-protein complex of each retarded band (Bound, nM) and free DNA probes (Free, nM) were plotted against the value of the bound forms. The dissociation constant ($K_d$) and $B_{max}$ were determined as 7.4 and 5.76 nM for the wild type receptor. The $K_d$ value of the mutant receptor could not be determined reliably because the calculated ratios of bound/free resulted in a large degree of scatter in the linear least squares plots.
In order to determine the magnitude of the effect caused by the EE(553,554)RG mutation for DNA binding, we tried to determine the binding affinity of this mutant and the wild type receptors. As shown in Fig. 5A, a constant amount of the TNT product was incubated with different amounts of labeled probes. The intensities of the free probes (free) and the specifically retarded bands (bound) were quantified using an imaging densitometer (Bio-Rad, model GS-700). Fig. 5B shows the plot of the data from the wild type receptors. The ratios of bound/free were plotted against the concentrations of the bound forms. From these plots, the $K_d$ was determined as 7.4 nM for the wild type. However, due to the very weak binding, the $K_d$ of the mutant receptor could not be calculated reliably. Therefore, it is concluded that TR2-11 binds DR5 as homodimers, C-terminal deletion mutants (for up to 49 amino acids) or the EE(553,554)RG mutant remain capable of binding to DR5, but at a much lower affinity, whereas mutation at the three leucine residues (537–539) completely abolishes the DNA binding property.

### A Trans-suppressive Activity Encoded within the LBD of TR2-11

To determine whether the suppressive activity of TR2-11 is transferable and to define the modular domain that is responsible for this activity, we transferred different portions of its DEF segment to the yeast GAL4-binding domain, resulted in pBD-TR2ΔN2, pBD-TR2ΔN2ΔC49, and pBD-TR2(518–590) (Fig. 6A), and tested their activity on the GAL4-tk-luc reporter as described under “Materials and Methods.” As shown in Fig. 6B, the expression of pBD-TR2ΔN2 resulted in a...
strong suppression (approximately 70%) of the basal activity of the luciferase reporter (column 2) as compared with the control (column 1). Interestingly, deletion of the C-terminal 49 amino acids abolished this trans-suppressive activity (column 3), whereas the C-terminal 73 amino acids alone had no trans-suppressive activity (column 4). Therefore, it is concluded that the suppressive activity of TR2-11 is transferable and requires the LBD including the C-terminal segment (the last 49 amino acids). It is still not known whether this C-terminal domain encodes a ligand-dependent activation function like the AF-2 of other receptors.

One mechanism for the transferable, suppressive activities of nuclear receptors involves receptor interactions with the corepressors such as N-CoR and SMRT (7, 8). Alternatively, heterodimerization with RXRs or RARs has also been implicated in the function of nuclear receptors (32). To determine whether TR2-11 employs such a mechanism for its suppressive activity, we examined the possibility of TR2-11 interaction with the known corepressor (N-CoR), RXRs, or RARs using the yeast two-hybrid interaction assay. As shown in Fig. 7, the DEF region of TR2-11 failed to interact with the known receptor interacting domain of the N-CoR (column 4), the RXR (column 8), and the RARs (column 8). The positive controls for interactions including TR2-11, N-CoR, RXRβ, and RARα were provided in columns 2, 3, 5, and 7, respectively. Therefore, it is concluded that the suppressive activity of TR2-11, although transferable and requiring the LBD, is not mediated by an interaction with the common receptor interacting domain of the N-CoR or heterodimerization with RXRs or RARs.

**DISCUSSION**

In this study, we have first dissected the functional domains involved in the suppressive activity of the wild type TR2-11 using a deletion strategy. We then produced mutant receptors by point mutations, and examined their biological activities and biochemical properties including receptor dimerization and DNA binding. From these mutation studies, it is concluded that receptor dimerization and DNA binding are generally important for the suppressive activity of TR2-11 and the C-terminal 49 amino acid sequence is involved in the suppressive activity, receptor dimerization and DNA binding. Fig. 8 shows the comparison of this region among TR2-11, RARα, RARγ, and RXRα. The three leucine residues (537–539) located on the predicted helix 10 are critical for receptor dimerization, DNA binding and suppressive activity. A glutamate residue located between helices 10 and 11, which is conserved among TR2-11, RXRα, RARα, and RARγ, is critical for the suppressive activity and DNA binding property but not for receptor dimerizing activity. This result indicates that the suppressive activity of TR2-11 is DNA binding-dependent but can be functionally uncoupled from its dimerizing activity. Furthermore, in the GAL4 reporter system, we demonstrate that TR2-11 encodes a transferable suppressive domain in its LBD and the C-terminal 49 amino acid residues are required for this trans-suppressive activity.

The slightly different effect of deleting 40 amino acids (TR2Δ40) between the artificial RARE-tk-luc and the native RAR-luc reporter systems is interesting. It is possible that the conformational change of this mutant receptor has a more drastic effect on the RARE-tk-luc than on the RAR-luc reporter. This is also supported by other data showing that the C-terminal 30–40-amino acid sequence plays an important role, as evidenced by the consequence of mutating the Glu-553 residue (see more discussion below). Despite this subtle variation, the overall changing pattern of mutant receptors agrees between the two reporter systems. Therefore, the physiological relevance of the DR5 used in RARE-tk-luc reporter (Fig. 1B) is strongly supported by the results using the native RARβ promoter (Fig. 1C) from which this particular DR5 is derived. RARβ directly responds to RA induction and has been established as one of the earliest responding retinoid receptors in eliciting biological responses, such as cell proliferation, differentiation, and apoptosis (33). In the testis, RA induction of RARβ expression is also shown to correlate with spermatogenesis (34). Our results would suggest that TR2 suppression of RA induction on the RARβ gene probably serves to fine tune the expression level of RARβ in developing germ cells.

The Kd of TR2-11 to the DR5 element is known to be much lower than that of the RAR/RXR heterodimers (35). Therefore, it has been suggested that competing for binding sites is the major mechanism mediating its suppressive activity on gene expression. In this current study, we demonstrate the presence of a trans-suppressive activity of TR2-11, in addition to its high affinity DNA binding. It is suggested that suppression by TR2-11 could occur at two different levels, i.e., by competition for DNA binding and by a transferable, active silencing activity. Since TR2-11 does not interact with RAR or RXR and the suppressive activity requires DNA binding, the sequestering mechanism can be excluded. The RNA polymerase II transcription component, i.e., TBP, has also been shown to interact directly with unliganded nuclear receptor (15, 16). However, we have failed to detect efficient interaction of TR2-11 with this molecule in the two-hybrid interaction assay (data not shown). Therefore, it is unlikely that TR2-11 suppresses the target genes by directly interfering with the basic transcription machinery such as TBP. However, interactions with other transcription factors cannot yet be excluded. Because its DEF segment can be transferred to GAL4-binding domain and remains suppressive, TR2-11 appears to be able to function in a manner...
similar to that of the unliganded thyroid hormone receptor and RAR. However, it does not interact with the known receptor-interacting domain of N-CoR. Therefore, the suppressive activity of TR2-11 is not mediated by interaction with these domains of N-CoR. It remains to be determined whether other portions of N-CoR can interact with TR2-11.

Efficient dimerization is important for the suppressive activity (as evidenced by the results of C-terminal deletions and the point mutations); however, dimerization alone is not sufficient for this suppressive activity (as evidenced by loss of suppressive activity of the glutamate mutant, which remains capable of dimer formation). As demonstrated also in other receptors (36, 37), the ninth heptad, which spans the conserved leucine residues (537–539), is important for both DNA-independent dimerization and the suppressive activity. The C-terminal helix of TR2-11 is important for its suppressive activity, particularly the C-terminal 30–40 amino acid residues. This region probably functions to maintain the integrity of the LBD and allows a specific conformation to be adopted for its high affinity DNA binding and suppressive activity, since a single Glu-553 mutation in this region completely abolishes the suppressive activity and drastically reduces its DNA-binding affinity. This is also supported by the fact that the chimera of GAL4-binding domain in this region completely abolishes the suppressive activity and drastically reduces its DNA-binding affinity. This is also supported by the fact that the chimera of GAL4-binding domain and TR2 C terminus requires this domain to exhibit the active silencing. It will be interesting to examine how the EE/ER mutation affects the receptor conformation.

The C-terminal 49-amino acid sequence includes part of the dimerization interface as well as the homologous region of the AF-2/4, which is essential for recruiting co-activators in other receptor systems (9–14). It remains unknown whether this domain of TR2-11 contains an AF-2-like ligand-dependent activation domain (as labeled with a question mark in Fig. 8). In other receptors, this motif participates in both transcriptional activation and the relief of silencing, as deletion of this domain results in failure to bind ligands and the receptor becomes a constitutive silencer due to the inability to release the corepressor (8, 38). It is suggested that a specific conformational change at the C terminus is induced by ligand binding, which subsequently causes the release of corepressors (39). It is possible that TR2-11 also employs such a mechanism, as a single glutamate mutation (Glu-553) is sufficient to release the suppressive activity. However, it remains to be determined whether TR2-11 interacts with any unknown corepressors and if any specific ligands are present for TR2-11 to function as an activator.

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