Convergence of two repressors through heterodimer formation of androgen receptor and testicular orphan receptor-4: A unique signaling pathway in the steroid receptor superfamily

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The androgen receptor (AR) binds to androgen response elements and regulates target genes via a mechanism involving coregulators. Here we demonstrate that the AR can interact with the testicular orphan receptor-4 (TR4) and function as a repressor to down-regulate the TR4 target genes by preventing the TR4 binding to its target DNA. Interestingly, the heterodimerization of AR and TR4 also allows TR4 to repress AR target gene expression. Simultaneous exposure to both receptors therefore could result in bidirectional suppression of their target genes. Together, these data demonstrate that the coupling of two different receptors, through the heterodimerization of AR and TR4, is a unique signaling pathway in the steroid receptor superfamily, which may facilitate further understanding of the complicated androgen action in prostate cancer or libido.

Nuclear receptors comprise the largest superfamily of eukaryotic transcription factors with more than 150 proteins identified. This superfamily includes intracellular receptors for steroid hormones, thyroid hormones, and retinoids, as well as a large number of orphan receptors for which regulatory ligands have not been identified. Regardless of whether transcriptional activity is controlled by binding of a ligand, each of these proteins must be capable of binding to a specific DNA sequence that identifies particular genes as targets for regulation. Protein-DNA interactions are mediated by the highly conserved DNA binding domain (DBD) that defines the nuclear receptor superfamily. Protein–protein interactions, necessary for the formation of homodimers and/or heterodimers, are mediated by an extensive C-terminal dimerization interface that is contained within the ligand binding domain (LBD) (1, 2). Accordingly, this superfamily can be divided into homodimeric receptors including receptors for androgen (AR), glucocorticoid (GR), estrogen (ER), and mineralocorticoid and a large diverse subfamily of nonsteroid receptors including receptors for thyroid hormone, retinoids, and vitamin D, as well as many orphan receptors for which the majority will heterodimerize with retinoid X receptor (RXR). These RXR heterodimers function as dynamic transcription factors in which one subunit influences the other's capacity to interact with ligand and cofactors (3–7). Another common heterodimer partner, short heterodimer partner, like RXR, can interact with various nuclear receptors and acts as a negative regulator of the nuclear receptor signaling pathway (8).

Like other nuclear receptors, the AR is a ligand-inducible transcription regulator that can activate or repress its target genes by binding to its hormone response element as a homodimer (9). It consists of four major functional domains including the DBD, the LBD, the hinge domain, and the N-terminal A/B activation domain (10, 11). It also contains two activation functions (AF) residing in the N-terminal A/B domain (AF-1) and the C-terminal end of the LBD (AF-2), respectively. AF-2 is responsible for hormone-dependent activation through recruitment of cofactors whereas AF-1 activity is constitutive and ligand independent. There are several AR coactivators that have been isolated by their interaction with either the N-terminal or LBD of AR and consequently enhance AR transactivation (12–16).

The human testicular orphan receptor-4 (TR4) originally was isolated from human prostate and testis cDNA libraries (17). The TR4 can modulate its target gene expression by forming TR4 homodimers and binding to AGGTCA direct repeat (DR) sequences in its target genes. Through this binding to its target DNA, we have demonstrated that the TR4 can modulate many signal transduction pathways, such as those involving retinoic acid (18), thyroid hormone (19), vitamin D_3 (20), and ciliary neurotrophic factor (21). Isolation of TR4 interacting proteins involved in modulating TR4-mediated gene regulation may extend our understanding of how the TR4 influences gene expression through cross-talk mechanisms, which do not require DNA binding.

During the search for novel proteins interacting with the TR4, we identified the AR as a receptor target for the TR4. In this report, we provide evidence for heterodimerization between the TR4 and AR, and consequently both receptors' influence on the other's target gene expression. The cross talk between the AR and TR4 not only extends the function of both receptors but also contributes to the understanding of the complex gene network controlled by the nuclear receptor superfamily.

Experimental Procedures

Glutathione *S*-Transferase (GST) Pull-Down Assay. GST-TR4 fusion protein and GST control protein were purified as instructed by the manufacturer (Amersham Pharmacia). Five microliters of *in vitro*-translated ^{35}S-methionine-labeled proteins was used to perform the pull-down assay as described (22).

Immunocytofluorescence. DU145 cells were seeded on two-well Lab Tek Chamber slides (Nalge) 18 h before transfection. One or two micrograms of DNA per 10^5 cells was transfected by the FuGENE6 transfection reagent (Boehringer-Mannheim). Transfected cells were treated with 100 nM dihydrotestosterone (DHT) or vehicle. Immunostaining was performed by incubating with anti-AR polyclonal antibody (NH27), anti-TR4 mAb (#15), and anti-ERα mAb (C-314, Santa Cruz Biotechnology), fol-

Abbreviations: AR, androgen receptor; TR4, testicular orphan receptor-4; DBD, DNA binding domain; LBD, ligand binding domain; GR, glucocorticoid receptor; ER, estrogen receptor; RXR, retinoid X receptor, DR, direct repeat; AF, activation function; DHT, dihydrotestosterone; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; PR, progesterone receptor; HBV, hepatitis B virus; MMTV, mouse mammary tumor virus; PSA, prostate-specific antigen.

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lowed by incubation with either fluorescein-conjugated goat anti-rabbit or anti-mouse antibodies (ICN) (23). The slides were photographed under 100-fold magnification by using a confocal microscopy.

Transient Transfection. Cells were routinely maintained in DMEM with 10% heat-inactivated FBS. The cells were transfected by using a modified calcium phosphate precipitation method (24) or SuperFect (Qiagen, Chatsworth, CA). To normalize the transfection efficiency, the β-galactosidase expression vector and pRL-TK were cotransfected in chloramphenicol acetyltransferase (CAT) assay and in dual-luciferase reporter assay system (Promega), respectively.

Electrophoretic Mobility Shift Assay (EMSA). The EMSA was performed as described (19). Briefly, the reaction was performed by incubating the ^{32}P end-labeled DR1 probe with *in vitro*-translated TR4 (1 μl) with or without an increasing amount of the AR (1, 2, or 4 μl). The EMSA incubation buffer was 10 mM Hepes, pH 7.9, 2% (vol/vol) glycerol, 100 mM KCl, 1 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT. For the antibody supershifted analysis, 1 μl of anti-TR4 mAb (#15) was added to the reaction. DNA-protein complexes were resolved on a 5% native gel. The radioactive gel was analyzed by autoradiography.

Northern Blotting Analysis. Total RNA from the DHT-treated transfected LNCaP cells was prepared by the ultracentrifugation method as described (18). The probe was obtained from exon 3 of the PSA gene and labeled with α-^{32}P dCTP.

Results and Discussion

***In Vitro* and *In Vivo* TR4 Interaction with AR.** Using a GAL4-TR4 fusion protein as bait in the yeast two-hybrid system, we were able to isolate several potential TR4-associated proteins. Sequence analysis and a GST-TR4 fusion protein pull-down assay confirmed that some of the candidates, such as the AR and the TR2, could physically interact with TR4 (Fig. 1*A*). In contrast, there was no interaction between the TR4 and RXR, another member of the steroid receptor superfamily (Fig. 1*A*). The AR was further characterized because of its profound effects on many androgen-related diseases. To more precisely map the regions in the AR that can interact with TR4, various AR deletion mutants were tested in the GST pull-down assay. As shown in Fig. 1*B*, TR4 can interact with three *in vitro*-translated [^{35}S]methionine AR deletion constructs: the N-terminal of AR (AR-N), the DBD of AR (AR-D), and the LBD of AR (AR-L). These results agreed with previous reports that coregulators were able to interact with both N-terminal and C-terminal domains of steroid receptors (25).

An immunocytofluorescence assay then was applied to determine the subcellular localization of the AR and TR4 in DU145 cells. Using specific anti-AR, anti-TR4, and anti-ERα antibodies, we found that unliganded AR was located mainly in the cytoplasm (Fig. 2*A*) and when bound to its cognate ligand, DHT, the AR signal moved to the nucleus (Fig. 2*B*). These data agreed with a previous report shown in COS cells (26). In contrast, TR4 and ER were detected as nuclear proteins even in the absence of exogenous ligand (Fig. 2 *C* and *D*, respectively). Interestingly, when AR and TR4 were cotransfected into DU145 cells, the majority of the AR signal could be detected together with TR4 signal in the nucleus, even in the absence of DHT (Fig. 2*E*). These data indicate that unliganded cytosolic AR moves into the nucleus once it is coexpressed with TR4 in DU145 cells. In contrast, when AR and ER were cotransfected into DU145 cells, the AR signal still remained mainly in the cytoplasm in a manner similar to that found when AR was transfected alone (Fig. 2*F*). The observation that unliganded AR can translocate into the nucleus in the presence of TR4 provides strong *in vivo* evidence that AR interacts specifically with TR4.

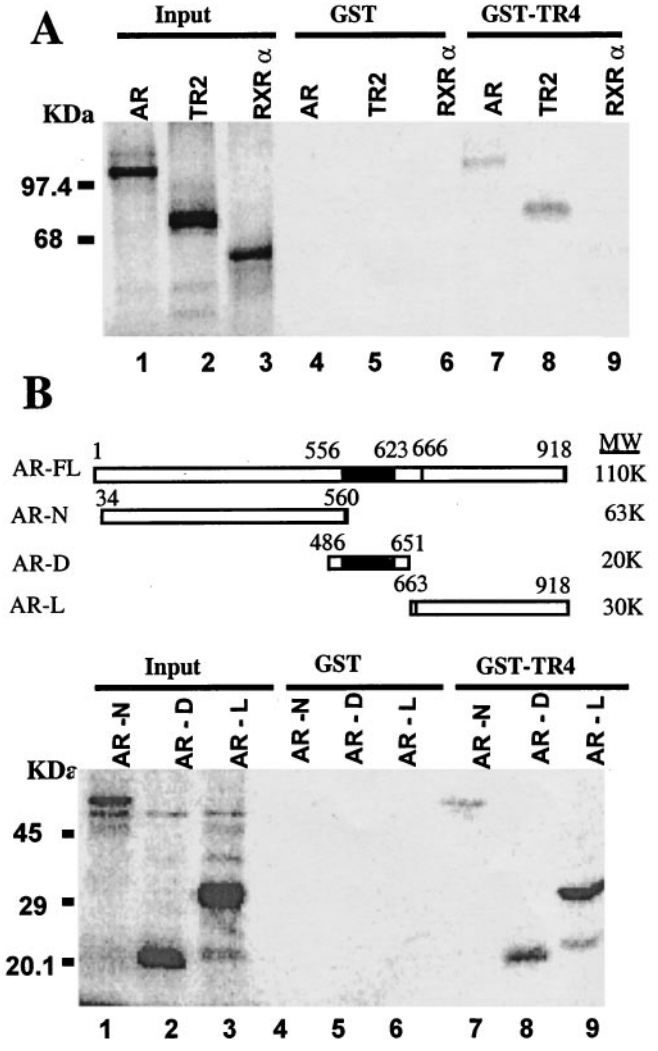

Fig. 1. Physical interaction between TR4 and AR. (*A*) The GST-TR4 fusion protein and GST control protein were purified as instructed by the manufacturer (Amersham Pharmacia). Five microliters of *in vitro*-translated ^{35}S methionine-labeled AR, TR2, and RXRα was incubated with the GST-TR4 or GST bound to glutathione-Sepharose beads in a pull-down assay as described. (*B*) Localization of the interaction domain within AR. Different AR deletion mutants, AR-N, AR-D, and AR-L, were *in vitro*-translated and incubated with the GST-TR4 in a pull-down assay. The input represents 20% of the amount of labeled protein used in the pull-down assay. The pull-down complex was loaded onto an 8% or 15% polyacrylamide gel and visualized by autoradiography.

This TR4 interaction with AR was further evaluated by the mammalian two-hybrid system assay. A near full-length human AR (amino acids 33–918) was fused to the transcriptional activator VP16 (VP16-AR) and then cotransfected with GAL4-DBD fused with TR4 LBD (GAL4-TR4E) and a GAL4-responsive luciferase reporter (PG5-Luc) in H1299 cells. As shown in Fig. 3*A*, either parental vector (pCMV-GAL4 or pCMV-VP16), VP16-AR, or GAL4-TR4E alone show a low background in the absence or presence of 1 nM DHT (lanes 1–6). Upon cotransfection of VP16-AR and GAL4-TR4E, a significant induction was observed by the addition of 1 nM DHT (Fig. 3*A*, lane 7 vs. 10), suggesting that DHT promoted the interaction between GAL4-TR4E and VP16-AR.

We used a modified mammalian one-hybrid system to avoid

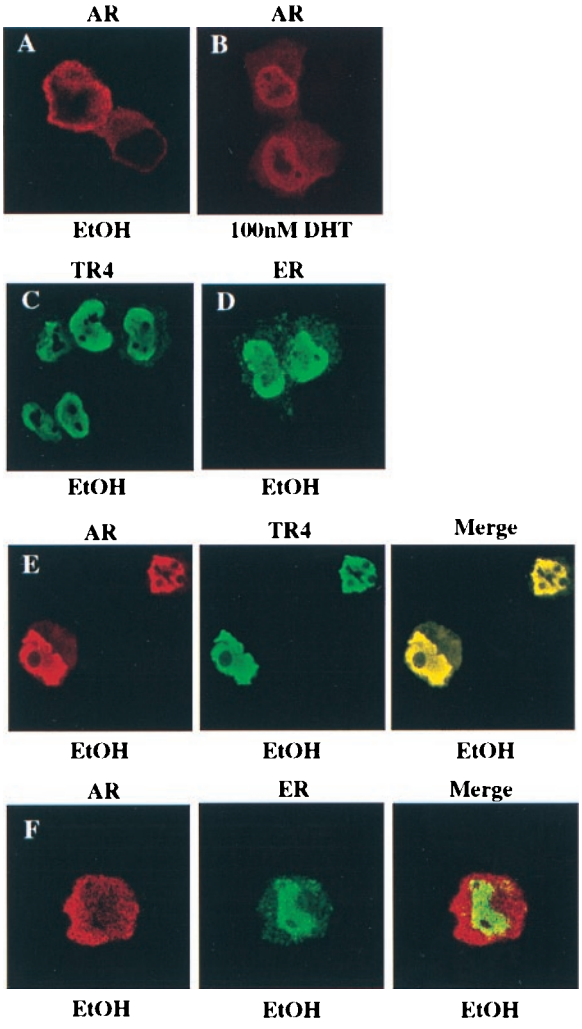

Fig. 2. Immunocytofluorescence detection of receptors in DU145 cells. DU145 cells were seeded on two-well Lab Tek Chamber slides (Nalge) 18 hr before transfection. One to two micrograms of DNA per 10^5 cells was transfected either with the AR (unliganded or liganded), TR4, or ER alone or in combination with the FuGENE6 transfection reagent (Boehringer-Mannheim). After 24 hr transfection, cells were treated with 100 nM DHT or ethanol. Immunostaining was performed by incubation with the anti-AR polyclonal antibody, anti-TR4 mAb, or anti-ERα mAb, followed by incubation with either fluorescein-conjugated goat anti-rabbit or anti-mouse antibodies (ICN). The red signal represents the AR, the green signal represents the TR4 or ER, and the yellow signal represents colocalization of the two signals in cotransfection of both receptors (the AR with TR4 or ER). Shown is the immunostaining of a single transfection of (*A*) unliganded AR, (*B*) 100 nM DHT treatment of the AR, (*C*) TR4, and (*D*) ER, and for cotransfection of (*E*) AR with TR4 and (*F*) AR with ER. The slides were photographed under 100-fold magnification by using confocal microscopy.

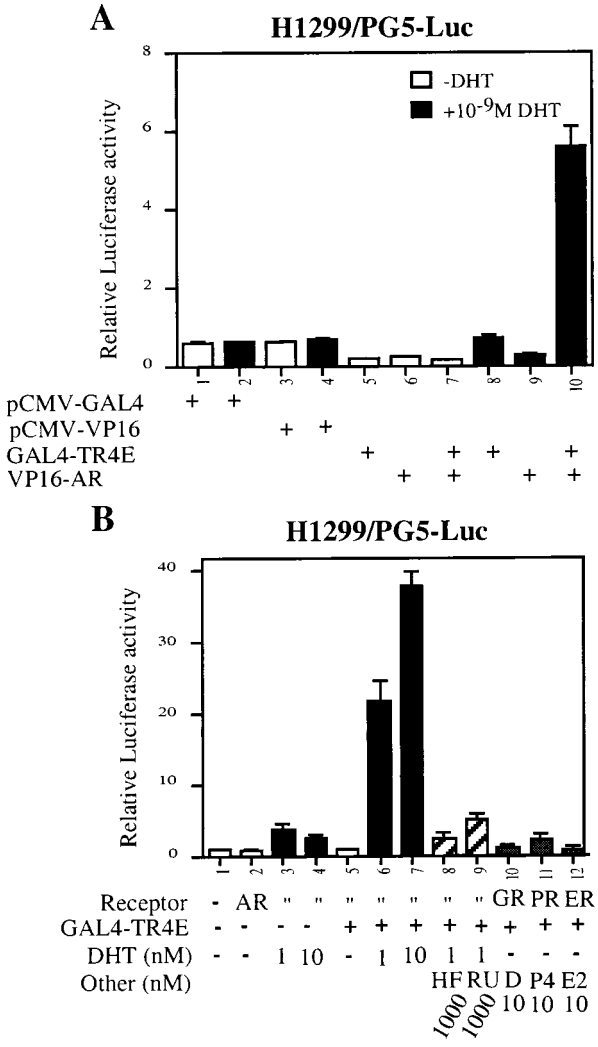

Fig. 3. (*A*) TR4 interaction with AR in the mammalian two-hybrid system. PG5-Luc (3.5 μg), the luciferase reporter gene containing five copies of GAL-DBD binding sites, was cotransfected with two fusion proteins, GAL4-TR4E, and VP16-AR. After 16–18 hr transfection, 1 nM DHT was added and ethanol was used in control groups. After 24 hr treatment, cells were harvested for dual luciferase assay. (*B*) TR4 interaction with AR in modified mammalian one-hybrid system. A total of 3.5 μg of *PG5-Luc* and 3 μg of GAL4-TR4E was cotransfected in the presence of 1 μg of pSG5AR (lanes 2–9), pSG5GR (lane 10), pSG5PR (lane 11), or pSG5ER (lane 12). Cells were treated as indicated. Transfection was performed by a modified calcium phosphate precipitation method. The pRL-TK plasmid was cotransfected for normalization of transfection efficiency.

the possibility that the DHT-dependent interaction between AR and TR4 is the result of artificial conformational changes created by the VP16-AR fusion protein in the mammalian two-hybrid system. A full-length AR (pSG5AR) was cotransfected with GAL4-TR4E and the PG5-Luc reporter in H1299 cells. As shown in Fig. 3*B*, transfection of pSG5AR alone showed only marginal DHT-dependent transactivation (lanes 2–4), whereas, cotransfection of pSG5AR and GAL4-TR4E showed a significant (20- to 40-fold) induction in the presence of 1–10 nM DHT (lanes 6 and 7). In contrast, no induction was observed when we replaced AR with other activated steroid receptors, such as GR, progesterone receptor (PR), or ER (Fig. 3*B*, lanes 10–12). Moreover, addition of 1 μM of antiandrogens, such as hydroxyflutamide or RU58841, could abolish the DHT-enhanced interaction between AR and TR4 (Fig. 3*B*, lanes 8 and 9).

The difference between DHT-dependent interaction detected in the mammalian one- or two-hybrid systems and DHT-independent interaction detected in the GST pull-down and the immunocytofluorescence assays might be caused by the involvement of the AF-1 ligand-independent interaction in the GST pull-down and the immunocytofluorescence assays vs. AF-2 ligand-dependent interaction in the mammalian one- or two-hybrid system. Taken together, results from the four experiments described in Figs. 1 and 3 provide strong evidence that AR and TR4 can interact in the presence or absence of DHT.

AR Represses TR4-Mediated Transactivation. The potential effects on transactivation by the AR-TR4 heterodimer formation then

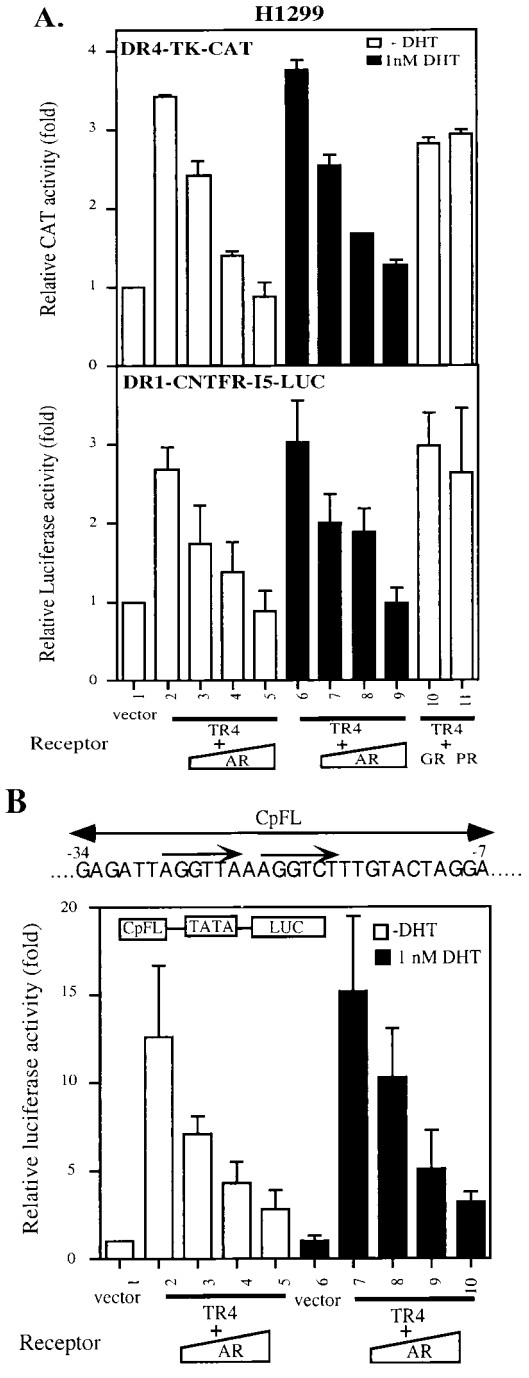

Fig. 4. AR repression of TR4-target gene expression. (*A*) AR repression of TR4-mediated DR4-TK-CAT and DR1-CNTFR-I5-LUC transcriptional activity. We cotransfected 500 ng of reporter plasmids (DR4-TK-CAT and DR1-CNTFR-I5-LUC) with 200 ng of pCMX-TR4 and increasing amounts of pCMV-AR (200, 600, and 1,200 ng), pSG5GR (1,200 ng), or pSG5 PR (1,200 ng) by using the SuperFect transfection kit (Qiagen). (*B*) AR repression of TR4-mediated HBV gene expression. The reporter plasmid CpFL(4)-LUC, which contains the HBV core promoter (Cp) sequence located between −34 and −7 (nucleotide coordinates 1751 and 1778 derived from the GenBank database) was shown above. The arrows indicate the DR1 motif in HBV core promoter. HepG2 cells were cotransfected with 1.5 μg of CpFL(4)-LUC reporter and 0.5 μg of pCMX-TR4, with increasing amounts of pCMV-AR (0.5, 2.5, and 5 μg) by modified calcium phosphate precipitation method. The relative reporter gene activities were compared with the CAT activities (or luciferase activities) with vector alone. To normalize the transfection efficiency, the β-galactosidase expression vector and pRL-TK were cotransfected in the CAT assay and in the dual-luciferase reporter assay system (Promega), respectively.

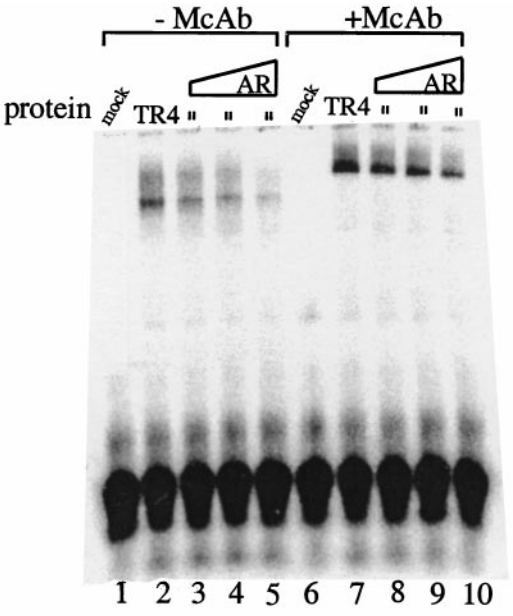

Fig. 5. Inhibition of TR4 DNA binding by AR. One microliter of *in vitro*-translated TR4 protein was incubated with increasing amounts of *in vitro*-translated AR (1 μl, 2 μl, and 4 μl) in EMSA reaction buffer [10 mM Hepes, pH 7.9/2% (vol/vol) glycerol/100 mM KCl/1 mM EDTA/5 mM $MgCl_2$/1 mM DTT] for 15 min. ^{32}P end-labeled DR1 was added into the protein mixture and incubated for 15 min before loading. For the antibody supershift assay, 1 μl of anti-TR4 mAb was added to the reaction and applied to a 5% native polyacrylamide gel. The radioactive gel was analyzed by autoradiography.

was tested through the use of a reporter assay: the full-length AR and TR4 in eukaryotic expression vectors (pSG5AR and pCMXTR4) were cotransfected with a CAT reporter containing a TR4-response element (DR4-TK-CAT) (19) in H1299 cells. As shown in Fig. 4*A*, the CAT activity induced by pCMXTR4 could be repressed significantly, in a dose-dependent manner by cotransfection of pSG5AR in the presence or absence of DHT. This repression of TR4 transactivation is AR specific, as other activated steroid receptors, such as GR or PR, have no suppressive effects (Fig. 4*A*, lanes 10 and 11). Similar results were obtained when we replaced the DR4-TK-CAT reporter with DR1-CNTFR-I5-LUC, another TR4 response element (21) (Fig. 4*A*).

We also investigated another potential TR4 target gene, which is located in the hepatitis B virus (HBV) enhancer II region (−34 to −7) containing a classic DR1 motif (27). As shown in Fig. 4*B*, TR4 can induce CpFL(4)-LUC activity, which is significantly decreased by cotransfection of the AR with TR4 in a dose-dependent manner. This finding suggested that the AR could regulate HBV gene expression through protein-protein interaction. Whether this regulation plays any role in the progression of hepatitis B or hepatoma, two diseases that have quite different levels of incidence between men and women (28), will be an interesting hypothesis to test in the future.

AR Prevents TR4 from Binding to Its Target DNA. The EMSA using ^{32}P-labeled DR1-TR4RE as a probe was applied to further dissect the mechanism of how the AR represses the TR4-mediated transactivation. As shown in Fig. 5, the specific TR4-DR1 band was decreased with the addition of increasing amounts of the AR (lanes 3–5). Furthermore, the intensity of the supershifted band formed by addition of anti-TR4 mAb to the TR4-DR1 complex also was decreased with the addition of increasing amounts of AR (Fig. 5, lanes 8–10). However, because of the unstable *in vitro*-translated AR protein, we failed to

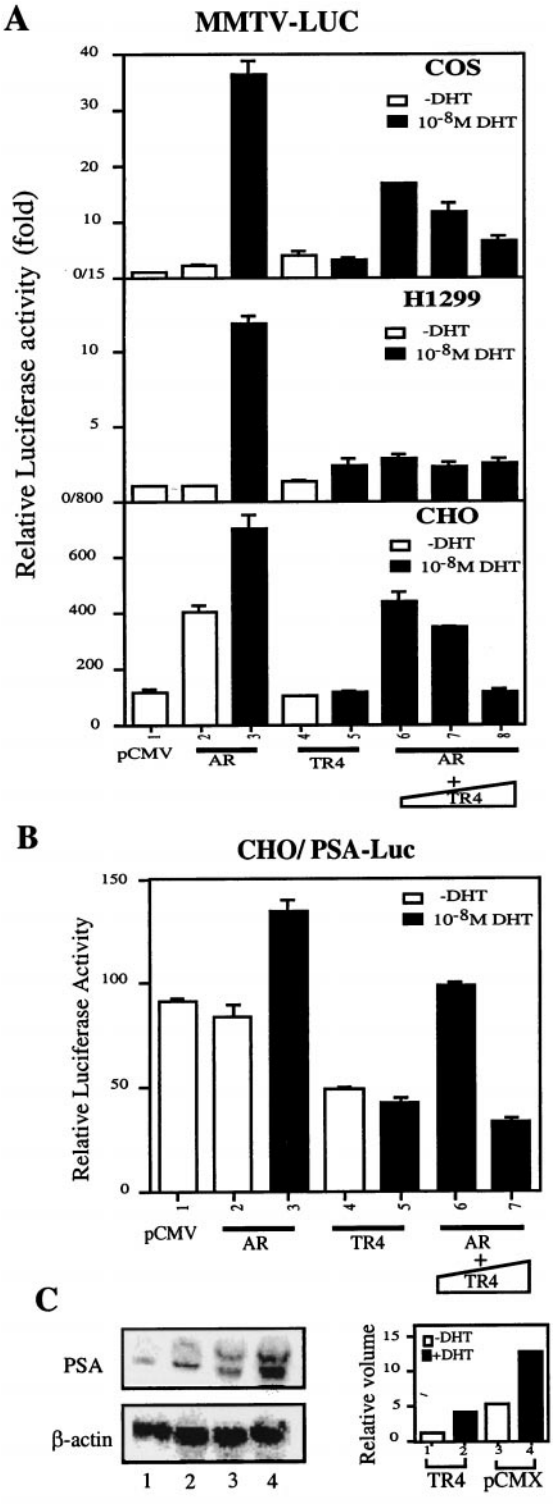

Fig. 6. TR4 repression of AR-mediated transcriptional activity. (*A*) Five hundred nanograms of MMTV-Luc was cotransfected with 40 ng of pCMV-AR (lanes 2 and 3) with increasing amounts of pCMX-TR4 (400, 800, and 1,200 ng). (*B*) Five hundred nanograms of PSA-Luc was cotransfected with 40 ng pCMV-AR (lanes 2 and 3) with increasing amounts of pCMX-TR4 (400 and 1,200 ng). After 24 hr transfection, cells were treated with 10 nM of DHT. After 16–18 hr incubation, cells were harvested for dual-luciferase reporter assay. (*C*) Northern blotting analysis of PSA transcripts in LNCaP cells. Total RNA (25 μg) from LNCaP cells, which were transfected with either pCMX-TR4 or pCMX vector by using SuperFect (Qiagen), was applied into a formamide RNA gel, transferred onto a nylon membrane, and hybridized with a ^{32}P-PSA gene fragment from the exon 3. β-actin was used as an internal control.

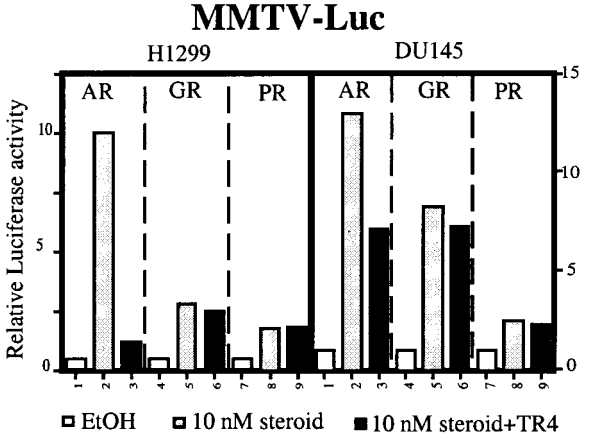

Fig. 7. The specificity of negative regulation on AR-mediated MMTV-luciferase activity by TR4. Three micrograms of MMTV-Luc was cotransfected with 4 μg of pCMX-TR4 in the presence of 1 μg of pSG5AR, pSG5GR, or pSG5PR by modified calcium-phosphate method. After 24 hr transfection, the cells were treated with 10 nM of synthetic steroids (DHT, dexamethasone, and progesterone). Dual-luciferase reporter assays were performed. pRL-TK was used to normalize the transfection efficiency.

demonstrate the interaction between AR, TR4, and androgen response element in the EMSA. It leaves the assumption that the repression of AR transactivation by TR4 also might be caused by prevention of DNA binding and/or quenching out the factors needed for AR transactivation. Together, these results suggested that the AR might be able to repress TR4-mediated transactivation by preventing TR4 from binding to its target DNA. As there is no extra supershifted band formed on adding AR to TR4-DR1 complex in Fig. 5, lanes 3–5, our data also may rule out the possibility of the formation of a transcriptional inactivated TR4-AR-DR1 complex.

TR4 Represses AR Target Gene Activation Both *In Vitro* and *In Vivo*. Like the TR4, the AR itself acts as a transcription factor to activate many androgen target genes. We were interested in investigating the potential negative-regulatory effects of the TR4 on AR-mediated transactivation. As expected, in COS, H1299, and Chinese hamster ovary cells, the AR activated mouse mammary tumor virus (MMTV) luciferase activity in a DHT-dependent manner (Fig. 6A, lanes 2 and 3), which then could be repressed by the addition of the TR4 (lanes 6–8). The TR4 by itself has no effect on the MMTV luciferase activity in the absence or presence of 10 nM DHT (lanes 4 and 5). Similar suppression effects also occurred when we replaced the MMTV-luciferase reporter with the prostate-specific antigen (PSA)-luciferase reporter, another AR target gene (Fig. 6*B*).

To rule out the potential artificial effects linked to transfected reporter assays, the expression of endogenous PSA (an androgen target that is widely used as a marker for prostate cancer progression) in LNCaP cells was measured by Northern blot analysis. As shown in Fig. 6*C*, the expression of the PSA transcript was induced about 2.5-fold after 24 hr of DHT treatment (lane 3 vs. 4). Addition of the TR4 can clearly repress the expression of the endogenous PSA transcript in either the absence (lane 1 vs. 3) or presence of 10 nM DHT (lane 2 vs. 4). The level of secreted PSA protein in the medium measured by ELISA also confirmed our conclusion (data not shown). This *in vivo* TR4-mediated suppressive effect strongly supports the above reporter assays and demonstrates that the TR4 may function as a repressor to negatively regulate the AR target genes expression.

TR4 Represses AR-Mediated Transactivation Specifically. As the GR and PR also can induce MMTV-luciferase reporter (11), we were interested in determining whether the TR4 also could repress GR- or PR-mediated transactivation. As shown in Fig. 7, whereas the AR, GR, and PR could induce MMTV-luciferase activity in the presence of their respective ligands in H1299 cells, cotransfection of the TR4 could repress only AR-mediated transactivation. Similar results were observed when we repeated the same experiments of the AR-mediated transactivation in DU145 cells. The TR4 represents a receptor that can heterodimerize with the AR with subsequent down-regulation of the AR transactivation.

Previous reports suggested that the RXR could function as a coactivator through heterodimer formation with the receptors for vitamin D, thyroid hormone, and peroxisome proliferator (29–32). The reverse repression effects of the vitamin D receptor, thyroid hormone receptor, and peroxisome proliferator receptor on RXR target genes, however, remain unknown. Our bidirectional repression effects through the AR and TR4 heterodimerization, therefore, represent a novel mechanism in the steroid receptor superfamily signaling pathway. The physiological significance of the AR-TR4 heterodimer is further supported by the similar expression pattern of both receptors in many tissues, such as the testis, hypothalamus, and prostate (17, 20, 33).

Two potential impacts of these findings are significant. First, the role of the AR in the modulation of androgen target genes may be expanded. In addition to activation of classic androgen target genes containing the androgen response elements (GGA/TACAnnnTGTTCT), AR also may signal through heterodimerization with the TR4, resulting in the repression of various TR4 target genes, which contain a consensus response element (AGGTCA) in a DR orientation ($(AGGTCA(n)_xAG-GTCA$, x = 0–6). Data from our gel shift assays showed that the binding preference of the TR4 for the natural TR4RE identified in various target genes was in the order of DR1 (CRBPII-TR4RE) (18) >DR2 (SV40-TR4RE) (34) >DR4 (TRE-TR4RE) (19) >DR5 (RAREβ-TR4RE) (18) >DR3 (VDRE-TR4RE) (20), with the IC_{50} varying widely from 0.023 ng to 2.0 ng. Among these TR4 target genes that could be suppressed by the AR, the HBV suppression might be especially interesting as it provides evidence that the AR may play a suppressive role in the HBV expression. Whether this regulation may contribute to the male-preference hepatitis B or hepatoma will be an interesting topic for future study. Second, we have demonstrated that the classic androgen-signaling pathway (A→AR→ARE) can be influenced by the TR4. This mechanism distinguishes between receptors (AR, GR, and PR) that share the same hormone response elements (found in the MMTV or other target genes), but also provides a potential target through which to block the androgen action. The long-term impact of these two events may be in providing us another approach in the design of the new generation of drugs with androgenic or antiandrogenic activity with which to treat androgen-related diseases.

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