The Orphan Nuclear Receptor TR2 Suppresses a DR4 Hormone Response Element of the Mouse CRABP-I Gene Promoter†

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ABSTRACT: The mouse orphan nuclear receptor TR2-11-f suppressed the expression of reporters fused to a hormone response element of the mouse cellular retinoic acid-binding protein I gene promoter. TR2-11-f was able to bind to a direct repeat with four nucleotides in the spacer (5′TGACCTTTGGGGACCT3′) located within this hormone response element as homodimers. The specificity of protein−DNA interactions was demonstrated by competition in gel retardation and antibody-mediated supershift reactions. The residues critical for TR2-11-f binding were mapped to both repeated sequences, whereas the spacer and the flanking sequences were less important. The K_d and B_{max} of TR2-11-f homodimer binding to this direct repeat were determined to be 2.6 nM and 0.012 nM, respectively. By using a yeast two-hybrid system, it was demonstrated that dimerization of TR2-11-f was mediated by its ligand-binding domain. The actions of TR2-11-f in regulating cellular retinoic acid-binding protein I gene will likely influence retinoic action and availability within the cells.

Nuclear receptors comprise a large family of transcription factors that regulate gene expression in a wide variety of biological processes, such as growth, differentiation, and development. Interactions of these receptors among themselves or with other transcription components alter the transcription machinery, thereby activating or suppressing their target gene expression (*1, 2*). The biological effects on their target genes are mostly mediated by binding to their cognate response DNA elements composed of repeated sequences in either direct or inverted orientations (*3*−*5*). Recently, a large number of nuclear receptors were cloned by homologous screening without the identification of specific ligands. They were termed orphan nuclear receptors.

The mouse homologue of the human orphan nuclear receptor TR2 (*6*) was cloned from a mouse embryonic cDNA library using the mouse retinoic acid receptor α ($RAR_α$)[1] as the probes in our previous studies (*7*). The exonic structure and chromosomal localization of the mouse gene, designated as *Tr2-11*, were determined (*7*). By aligning the cDNAs to the genomic sequences, two types of TR2-11 transcripts were identified. One encoded 590 amino acid residues translated from 13 exons, designated as TR2-11-f (full length); the other was generated by alternative splicing at the intron following the DNA-binding domain and encoded only 256 amino acid residues due to the introduction of an early termination codon from the unspliced intron sequence. The truncated receptor was designated as TR2-11-t. Proteins translated from these two types of mRNAs were confirmed by Western blot analyses, and their biological functions were first examined in a reporter system containing a direct-repeat 5 (DR5)-type retinoic acid response element (RARE) (*8*). The expression of this gene was specific to the male reproductive system and developing mouse embryos, but the two isoforms were expressed differentially among testicular cell types. TR2-11-f was most highly elevated in advanced germ cells such as pachytene spermatocytes and round spermatids, whereas TR2-11-t was expressed only in somatic cells (*9*). This gene was also specifically expressed in a mouse embryonal carcinoma cell line, P19, and its expression was rapidly induced by retinoic acid (RA) (*8, 9*).

The specificity of TR2-11-f expression in advanced germ cells (*9*) and its strongly suppressive effects on RARE-containing reporters (*8*) prompted us to investigate the possibility of a regulatory role of TR2-11-f on the expression of the mouse cellular retinoic acid-binding protein I (*CRABP-I*) gene, a gene highly expressed in early germ cells and suppressed in advanced germ cells. This gene promoter contained a DNA sequence characteristic of a hormone response element which consisted of a DR4 (*10*). It was believed that CRABP was involved in RA metabolism or its mobilization into the nuclei, thereby controlling the amount of RA molecules available to their nuclear receptors, RARs, and retinoid receptors X (RXRs) (*11*−*13*). Two types of CRABPs were cloned. The type II protein (CRABP-II) was specific to the skin (*14, 15*) and was directly induced by RA via an RARE located in its promoter region (*16*). In contrast, the type I protein (CRABP-I) was ubiquitously expressed in animal tissues but was highly elevated in several tissue/cell types which were more sensitive to a disturbance in vitamin A homeostasis, such as premeiotic germ cells (*17*),

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[1] Abbreviations: RA, retinoic acid; CRABP, cellular retinoic acid-binding protein; RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, retinoid receptor-X; TR2-11-f, TR2-11 full-length protein; TR2-11-t, TR2-11 truncated protein; $T_3R_{β1}$, thyroid hormone receptor-β1; lacZ, β-galactosidase; bp, base pair(s); kb, kilobase(s); DR, direct repeat.

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the pigment epithelium of the eyes, and certain neuronal cells (*18*−*21*). In addition, CRABP-I was regulated by complicated mechanisms. For instance, various signals such as serum, protein kinases (*22*), DNA methylation (*23*), RA (*24*), and thyroid hormones (*25*) have been found to directly affect the expression of this gene in both cultured cells and animals. In P19 cells, CRABP-I expression was suppressed, whereas TR2-11 was induced, rapidly by RA treatment (*8*, *9*, *25*). Because of the complementary patterns of CRABP-I and TR2-11-f expression in the testis and P19 cells and the presence of DR4 within the hormone response element in the *CRABP*-I promoter, a regulatory role of TR2-11 on *CRABP*-I gene expression by binding to this DR4 was suspected.

In this study, we demonstrated that expression of TR2-11-f, but not of TR2-11-t, exerted a strongly suppressive activity on reporter genes containing the DR4 of CRABP-I promoter. TR2-11-f, but not TR2-11-t, preferentially formed homodimers on this repeated sequence, and the residues in both repeats were crucial for protein binding. Using the yeast two-hybrid system to examine protein−protein interactions, we further demonstrated that the ligand-binding domain of this nuclear receptor was responsible for the formation of receptor homodimers.

EXPERIMENTAL PROCEDURES

Reporter and Expression Vector Constructs. The CRABP-lacZ reporters with the DR4 retained (the −1046 construct) or deleted (the −993 construct) in the context of its natural promoter sequence were as described (*10*). The heterologous reporter was constructed by fusing the 54 bp fragment (positions −1046 to −993) to the upstream region of the RAR_β minimal promoter driving a luciferase cassette (RAR-luc), designated as DR4-RAR-luc. Because, in our preliminary studies, TR2-11 expression was strongly inhibitory to other routinely used promoters such as SV40 and thymidine kinase promoters, the RAR_β minimal promoter was chosen to test the function of DR4 in a heterologous promoter. The expression vectors for TR2-11-f and TR2-11-t were constructed by inserting each coding sequence, with its natural translation initiation signal, into a cytomegallovirus (CMV) expression vector (*26*), and were designated as CMV-TR2-f and CMV-TR2-t, respectively. To express proteins for gel retardation experiments, the coding sequences of TR2-11-f and TR2-11-t were each inserted into a T7 expression vector (pSp73; Promega, Madison, WI) in a sense orientation for *in vitro* transcription and translation.

Mammalian Cell Transformation and Determination of Reporter Activities. Cos-1 cells were maintained as described (*10*). To determine the biological activities of TR2-11 on the DR4 placed in its natural promoter, Cos-1 cells were plated in 6-well plates (1.5×10^5 cells/well) and cotransfected with one of the CRABP-lacZ reporters (−1046 or −993 construct, 0.5 μg each), the TR2-11 expression vector (0.1 μg), and an internal control vector (0.1 μg, RAR-luc, see the following) using the calcium phosphate precipitation method as described (*22*). Because both the SV40 and thymidine kinase promoters were strongly suppressed by the expression of TR2-11-f, the commonly used internal control vectors such as pGL3 (Promega) or tk-luc (*10*) could not be used. Our preliminary studies showed that the RAR_β minimal promoter was not suppressed by these nuclear receptors in Cos-1 cells. Therefore, reporters for internal control in cotransfection experiments were made by inserting the RAR_β minimal promoter to a promoterless lacZ or luciferase cassette, designated as RAR-lacZ and RAR-luc, respectively. The LacZ (OD_{420}) and luciferase (relative luciferase unit, RUL) activities were determined between 24 and 40 h to obtain specific reporter activities (OD_{420}/RUL) as described previously (*25*). To determine the biological activities of TR2-11 on the DR4 transferred to a heterologous promoter, Cos-1 cells were transformed with the heterologous reporter (DR4-RAR-luc) or its parental vector (RAR-luc), the TR2-11 expression vector, and an internal control RAR-lacZ. The specific reporter activity (RUL/OD_{420}) was determined by normalizing the luciferase activity to the internal control lacZ activity. The effects of TR2-11 expression vectors on the DR4 fused to a heterologous promoter were represented as the ratio of the specific reporter activity of DR4-RAR-luc ($A_{DR4-RAR-luc}$) to that of the parental reporter ($A_{RAR-luc}$) in the presence of the TR2-11. For all the assays, triplicate cultures were used in each experiment, and three to five independent experiments were conducted to obtain the means and standard errors of the mean (SEM).

Gel Retardation Assay. Gel retardation experiments were conducted by using in vitro synthesized proteins as described previously (*25*). In vitro transcription and translation was conducted by using the TNT T7-Coupled Reticulocyte Lysate System (Promega). A chicken antibody was generated against TR2-11-f expressed in *E. coli*. The antibody was purified using chloroform−poly(ethylene glycol) (*27*), absorbed with untransformed *E. coli* lysate, and tested on a Western blot. For supershift experiments, the purified antibody, or the preimmune serum, was added to protein−DNA complex and incubated for 30 min at room temperature. The reaction mixtures were then analyzed similarly on polyacrylamide gels. To conduct competition experiments, wild-type or mutated DNA fragments were prepared and used as cold competitors in protein−DNA reactions.

Techniques for the Yeast Two-Hybrid System. Yeast strain YRG-2 containing a lacZ reporter controlled by three copies of GAL4-binding sites, as well as the expression vectors for the bait (pBD) and the prey (pAD), was purchased from Strategene (La Jolla, CA). The bait was prepared by inserting the ligand-binding domain of TR2-11-f (amino acid residues 167−590) (*7*) into the *Sal*I site, in-frame with the binding domain of GAL4 (amino acid residues 1−147) into the yeast vector pBD, designated as pBD-TR2. The prey was prepared by inserting a slightly shorter ligand-binding domain of TR2-11-f (amino acid residues 238−590) into the *Eco*RI and *Xho*I sites, in-frame with the GAL4 activation domain (amino acid residues 761−881) of pAD, designated as pAD-TR2. Yeast were cotransformed with both the bait and the prey vectors and plated in selective medium. The control vectors included a prey vector containing the SV40 large T-antigen (amino acid residues 84−708) inserted in the pAD (pAD-SV40), a bait (pBD-lamC) containing the human lamin C (amino acid residues 67−230) inserted in the pBD, and a second bait (pBD-p53) containing the p53 (amino acid residues 72−390) inserted in the pBD. Following transformation, single colonies were isolated for determination of lacZ activities according to a formula that defined lacZ units as $1000 \times OD_{420}/t \times V \times OD_{600}$. The OD_{420} value was obtained from enzymatic reaction with *o*-nitrophenyl β-D-galactopyranoside as the substrate. The

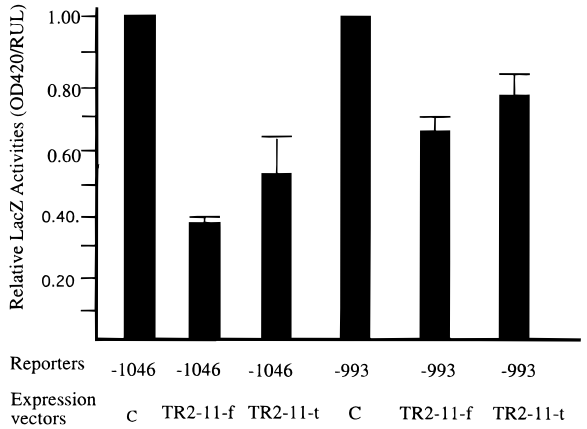

FIGURE 1: Suppression of CRABP-lacZ reporters by TR2-11 receptors in Cos-1 cells. Cos-1 cells (1.5×10^5) were transfected with one of the reporters, one of the expression vectors, and an internal control, RAR-luc. LacZ and luciferase activities were determined at 30 h, and each relative lacZ activity was determined by normalizing the lacZ activity (OD_{420}) to the luciferase activity (RUL) and represented as OD_{420}/RUL.

t value (in minutes) was the incubation time for the enzymatic reaction, and the *V* value equaled the value of 0.1 mL × concentration factor. The OD_{600} value was derived from the absorbancy of 1 mL of yeast culture at the 600 nm wavelength.

RESULTS

A Strongly Suppressive Activity of TR2-11-f on the DR4 of the Mouse CRABP-I Gene. Previously, we have constructed a series of in-frame reporter genes by inserting the lacZ structural gene into the sixth codon of the mouse CRABP-I genomic segments, followed by systematic deletions from the 5′ upstream region (*10*). By using the P19 cell line as a model system, in which CRABP-I expression was specifically regulated, a negative hormone response element was identified which contained a DR4 overlapped with a DR5 within a 54 bp region between positions −1046 and −993 (*10*). In cotransfection experiments, we first observed a strongly suppressive effect of TR2-11-f on the reporter containing the 3 kb 5′ upstream region of the CRABP-I gene in P19 cells. The truncated receptor TR2-11-t also exerted slight suppression on these reporters. Subsequently, it was found that TR2-11-f strongly suppressed both the parental reporter (CRABP-lacZ) and its deletion (the −1046 construct) that retained this response element, but affected a reporter deleted in this element (the −993 construct) much less efficiently. It was suggested that the region responsible for most of the suppressive effect of TR2-11-f resided in this response element. To confirm the suppressive effect of these nuclear receptors in a cleaner cellular background, the experiments were repeated in Cos-1 cells. Cos-1 cells were cotransfected with one of the two reporters (the −1046 construct containing the DR4 and the −993 construct deleted in the DR4), one of the expression vectors (TR2-11-f, Tr2-11-t, or a control vector), and the internal control vector RAR-luc. As shown in Figure 1, TR2-11-f inhibited the expression of the −1046 reporter approximately 60% (column 2) and the −993 reporter approximately 35% (column 5). TR2-11-t inhibited both reporters approximately 40% (columns 3 and 6). Thus, a suppressive effect of both TR2-11-f and TR2-11-t on the CRABP-I promoter was also detected in Cos-1 cells, and an additional

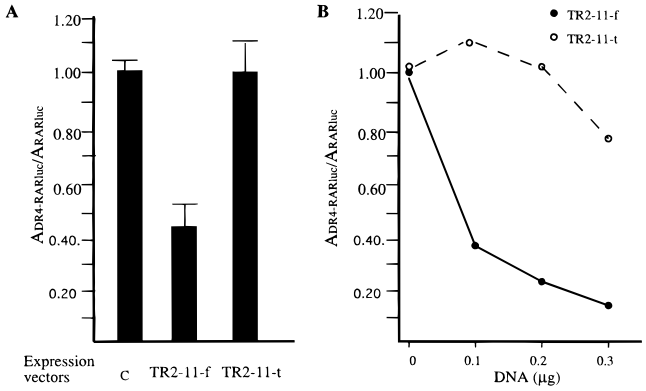

FIGURE 2: TR2-11-f suppressing the DR4 placed in a heterologous promoter. (A) Various suppressive effects of TR2-11 receptors. Cos-1 cells were cotransfected with one of the reporters (RAR-luc or DR4-RAR-luc), one of the TR2-11 expression vectors (control, TR2-11-f, or TR2-11-t), and an internal control (RAR-lacZ). LacZ and luciferase activities were determined at 30 h to obtained the specific reporter activity represented as RUL/OD_{420}. The specific reporter activity of the parental reporter (RAR-luc) was designated as $A_{RAR-luc}$, and the specific reporter activity of the heterologous reporter (DR4-RAR-luc) was designated as $A_{DR4-RAR-luc}$. The degree of suppression was represented by dividing the $A_{DR4-RAR-luc}$ value by the $A_{RAR-luc}$ value for each expression vector. The results from three independent experiments are shown here. (B) Dose-dependent suppression by TR2-11-f. Cotransfection was conducted in Cos-1 cells with one of the reporters and the indicated amounts of TR2-11-f expression vectors. The degree of suppression was represented as described in part A and plotted against the amounts of TR2-11-f expression vector.

negative activity of TR2-11-f on the DR4 of the CRABP-I promoter was confirmed (by comparing 60% suppression of the −1046 reporter to 35% suppression of the −993 reporter).

To determine if the negative effects of TR2-11 on this DR4 could be reproduced in a heterologous promoter system, we designed another reporter by fusing this DR4 to a luciferase reporter controlled by the minimal promoter of the RAR_β gene, RAR-luc (*28*), which by itself was not suppressed by this nuclear receptor expression in Cos-1 cells. This reporter was designated as DR4-RAR-luc. Cos-1 cells were cotransfected with one of the reporters (DR4-RAR-luc or RAR-luc), together with an expression vector (TR2-11-f, Tr2-11-t, or the control vector) and a lacZ vector for transfection control (RAR-lacZ). The specific reporter activities were determined by normalizing luciferase activities to lacZ activities and are represented as RUL/OD_{420}. The results of three independent experiments are shown in Figure 2A. TR2-11-f, but not TR2-11-t, suppressed this heterologous reporter activity approximately 50% at the amount of 0.1 μg. We then determined if the suppression of this reporter by TR2-11-f was dose-dependent. As shown in Figure 2B, a dose-dependent suppressive effect of TR2-11-f was observed within the concentration range of 0.1−0.3 μg. At a higher concentration (0.4 μg), the reporter activity was suppressed to a background level (data not shown), possibly due to toxic effects by overexpression of this receptor. Very differently, TR2-11-t exerted a slightly suppressive effect on this heterologous reporter only at high concentrations (0.3 μg or higher). Thus, it was concluded that TR2-11-f suppressed reporter genes containing the DR4 sequence (from −1046 to −993 positions) derived from the mouse *CRABP*-I gene promoter, in the context of natural or heterologous promoters. In contrast, TR2-11-t had no significant effect on this DR4.

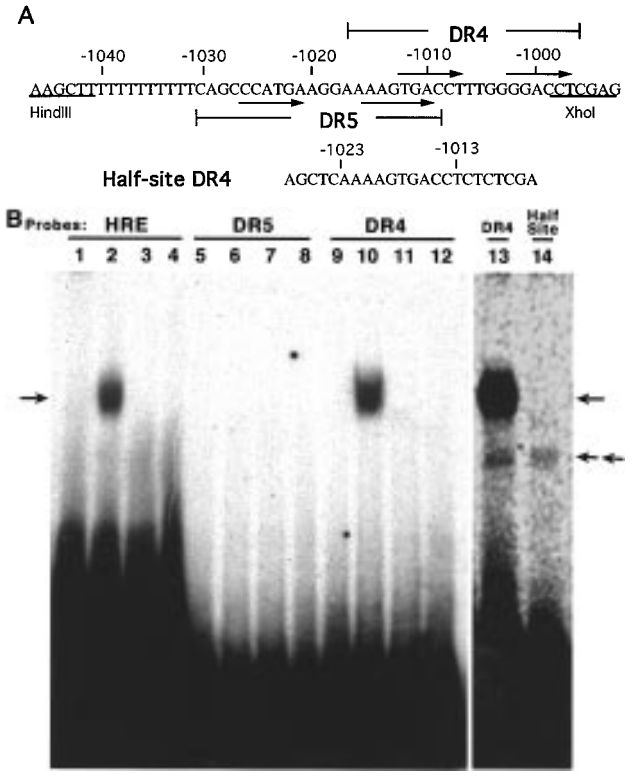

FIGURE 3: Specific protein binding of DR4 by TR2-11-f. (A) Nucleotide sequence of the hormone response element. The DNA fragments used as the probes were labeled on the sequence, including the entire hormone response element, the DR5, the DR4, and the half-site DR4. (B) Gel-retardation experiments using various DNA fragments derived from this hormone response element as the probes and TR2-11-f proteins synthesized from in vitro transcription/translation reactions. Arrows indicate the specifically retarded band, representing homodimers of TR2-11-f binding to the probes. The entire response element (HRE), lanes 1−4; DR5 probe, lanes 5−8; DR4 probe, lanes 9−13; half-site probe, lane 14. Lanes 1, 5, 9, probe alone; lanes 2, 6, 10, 13, 14, TR2-11-f; lanes 3, 7, 11, TR2-11-t; lanes 4, 8, 12, reticulocyte lysate control. A double arrow indicates the position of a faster migrating band, representing monomer binding of TR2-11-f to the DR4 (lane 13) or the half-site (lane 14) from CRABP-I promoter.

Protein−DNA Interaction of TR2-11 with the HRE of CRABP-I. To examine if this DR4 fragment (sequence shown in Figure 3A) could interact with TR2-11 proteins, gel retardation experiments were conducted by using, first, the 54 bp fragment as the probes, as shown in Figure 3B (lanes 1−4). As expected, this fragment was specifically bound by TR2-11-f (lane 2), but not by TR2-11-t (lane 3) or the reticulocyte lysate alone (lane 4). Within this sequence, two overlapping DRs were present, one of the DR5 type and the other of the DR4 type. To define the sequence responsible for protein binding to this element, the DR5 and DR4 sequences were individually labeled as probes and tested in similar gel retardation experiments. As shown in Figure 3B, the DR5 sequence could not be bound by either TR2-11-f or TR2-11-t (lanes 5−8), whereas the DR4 sequence could be bound by TR2-11-f (lane 10) but not by the control lysate (lane 12) or TR2-11-t (lane 11). Thus, the sequence responsible for TR2-11-f binding was located in the DR4 repeats of this sequence. To determine if the retarded band was due to binding by dimeric or monomeric TR2-11-f proteins, a DNA fragment containing a half-site of the DR4 flanked by nonspecific sequences at both sides (sequence shown in Figure 3A, labeled as half-site DR4) was used in similar gel retardation experiments. The bound patterns, by using either the DR4 or the half-site DR4 probes, were compared on the same gel as shown in lanes 13 and 14 of Figure 3B. The retarded band using the DR4 as probes migrated at a higher position (lane 13) than the retarded band using the half-site DR4 as probes (lane 14). In addition, the intensity of the faster migrating band (in the lane using a DR4 half-site as the probes) was much weaker than the slower migrating band seen in the lane using the complete DR4 as the probes. The weaker and faster migrating band represented retarded probes bound by monomeric TR2-11-f to the DR4 half-site, whereas the stronger and slower migrating band represented the retarded DR4 probes bound by the dimeric proteins. From the relative intensity, it was also concluded that monomeric TR2-11-f binding was much weaker than dimeric TR2-11-f binding to this DR4 sequence.

To confirm the specificity of the TR2-11-f interaction with this DR4, two experiments were further conducted, one utilizing wild-type or mutated DNA fragments as competitors in gel retardation experiments and the other utilizing a specific antibody to TR2-11-f in supershift experiments. The DR4 DNA fragments were labeled as probes and subjected to similar protein−DNA interactions in the presence of a 100× excess of unlabeled (cold) DNA fragments of the wild-type sequence or the mutated sequences (sequences shown in Figure 4A), and the results are shown in Figure 4B. Without cold DNA fragments, the labeled DR4 was bound by TR2-11-f, resulting in a specifically retarded major band (lanes 2 and 8) which was competed out by incubation with the cold, wild-type DR4 fragments (lanes 3 and 9). To determine specific residues of this DR4 critical for protein−DNA interaction, mutated DNA fragments were used as competitors as shown in lanes 4, 5, 6, 7, and 10. Mutations in either of the two repeats (m2, m3, or m5) resulted in failure to compete with the wild-type DNA−protein interactions (lanes 5, 6, and 10, respectively), whereas mutations in either the flanking (m1) or the spacer (m4) sequences retained the ability to compete in DNA−protein interactions (lanes 4 and 9, respectively). From these experiments, it was concluded that TR2-11-f specifically interacted with the DR4 DNA fragments and the residues of both repeats were critical to this protein−DNA interaction. Mutations in the flanking or spacer regions had no significant effects on protein−DNA interactions.

A polyclonal chicken antibody against TR2-11-f proteins expressed from *E. coli* was generated. The purified antibody was first tested on a Western blot as shown in Figure 5A. Both TR2-11-f and TR2-11-t were recognized by this polyclonal antibody (lane 2) as compared to the control culture which contained no expression plasmid (lane 1). By using this chicken antibody, supershift experiments were conducted as shown in Figure 5B. The preimmune serum (lane 3) had no effects on the migration of the retarded bands as compared to the control reaction (lane 2, no antiserum added). In the presence of the antibody, a band at a higher position appeared (lane 4), indicating a specific antibody-mediated supershift of this protein−DNA complex. Therefore, the specificity of the TR2-11-f interaction with the DR4 sequence was further confirmed.

Binding Affinity of TR2-11-f to the DR4. To determine the binding affinity of TR2-11-f to this DR4 sequence, gel-shift experiments were conducted by incubating a constant amount of *in vitro* synthesized TR2-11-f with different

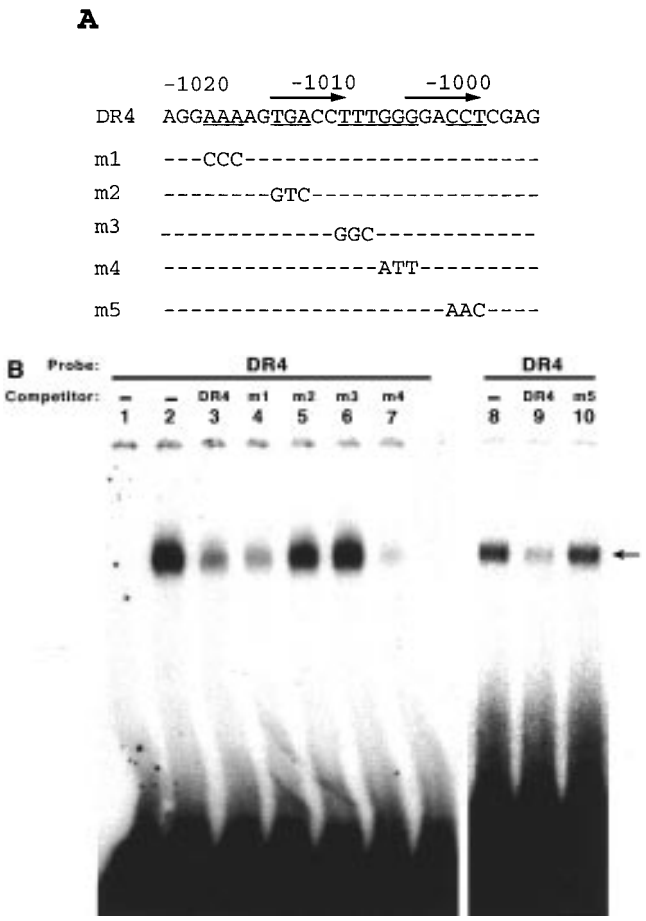

FIGURE 4: Effects of mutations in the DR4 sequence on TR2-11-f binding. (A) The wild-type (DR4) and mutated sequences m1−m5 are shown. Mutated sequences are shown under the wild-type sequence in which mutations were underlined. (B) Effects of mutations in the DR4 sequence on TR2-11-f binding. The wild-type DR4 fragments were labeled as the probes, and cold DNA fragments (wild-type DR4, and mutants m1, m2, m3, m4, and m5) were added as the competitor as indicated. TR2-11-f prepared from in vitro transcription/translation was added to each reaction. Lane 1, a control reticulocyte lysate without TR2-11-f; lanes 2 and 8, positive control reactions without cold DNA fragments; lanes 3−7, 9, 10, competitors added. The arrow indicates the specifically retarded band.

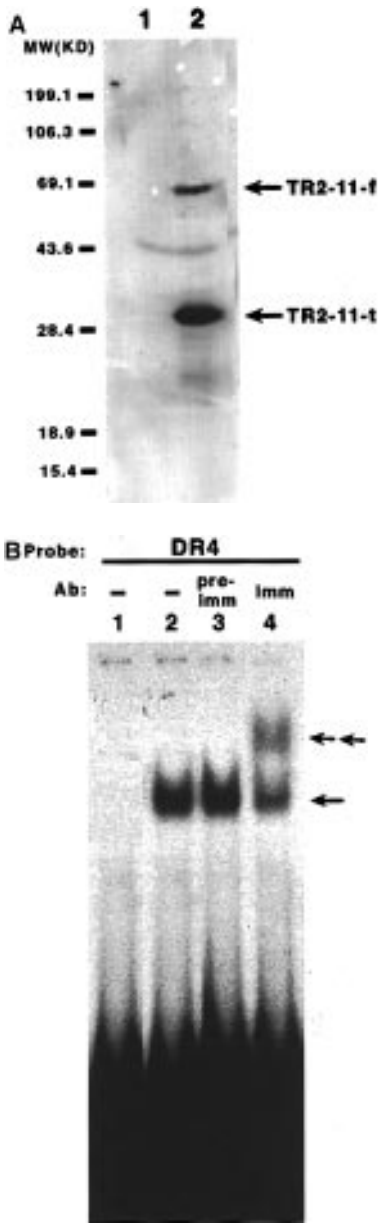

FIGURE 5: Antibody-mediated supershift experiments. (A) A Western blot showing the specificity of antibody raised against TR2-11-f. *E. coli* lysates were prepared from untransformed culture (lane 1) or a mixture of cultures transformed with TR2-11-f and TR2-11-t expression vector. The positions of TR2-11-f and TR2-11-t are indicated with arrows. (B) Supershift experiments. The wild-type DR4 fragments were labeled as the probes (lane 1). TR2-11-f was added to the probes (lanes 2−4) in the presence of preimmune IgY (lane 3) or immune IgY (lane 4). The arrow indicates the band bound by homodimers, and the double arrow indicates the position of the antibody-mediated supershift.

amounts of labeled probes ranging from 0.1 to 2.0 ng. The protein−DNA mixtures were then resolved on a polyacrylamide gel as shown in Figure 6A. The intensities of the unbound probes (labeled as free) and the major retarded bands representing dimeric protein binding (labeled as bound) were quantitated using an imaging densitometer (Bio-Rad, Model GS-700). The ratios of bound/free were plotted against the concentrations of the bound forms as shown in Figure 6B. The K_d and B_{max} values were determined as 2.6 nM and 0.012 nM, respectively.

The Ligand-Binding Domain of TR2-11-f Was Responsible for Its Dimer Formation. From gel retardation experiments, it was concluded that TR2-11-f, but not TR2-11-t, was able to bind to the DR4 sequence of the CRABP-I promoter, primarily as homodimers. Since the truncated form receptor, TR2-11-t, was identical to TR2-11-f at the N-terminal and DNA-binding domains but lacked the ligand-binding domain, it was suspected that the ligand-binding domain might have contributed to protein−protein interactions for TR2-11-f dimer formation. To examine this possibility, the yeast two-hybrid system based protein−protein interaction test was conducted. The bait vector (pBD-TR2) contained the entire ligand-binding domain (amino acids 167−590) of TR2-11-f, and the prey vector (pAD-TR2) contained a slightly shorter sequence (amino acids 238−590). As shown in Figure 7, in yeast cotransformed with different combinations of the bait and the prey, lacZ reporter was activated only in the positive control (pBD-p53/pAD-SV40) and in the culture transformed with both the pBD-TR2 and the pAD-TR2. In all the negative controls, including cotransformation with pBD-lamC/pAD-SV40, pBD-TR2/pAD-SV40, and pBD-lamC/pAD-TR2, no activity was detected. Thus, it was concluded that the sequence within the ligand-binding domain was able to mediate dimerization of TR2-11-f

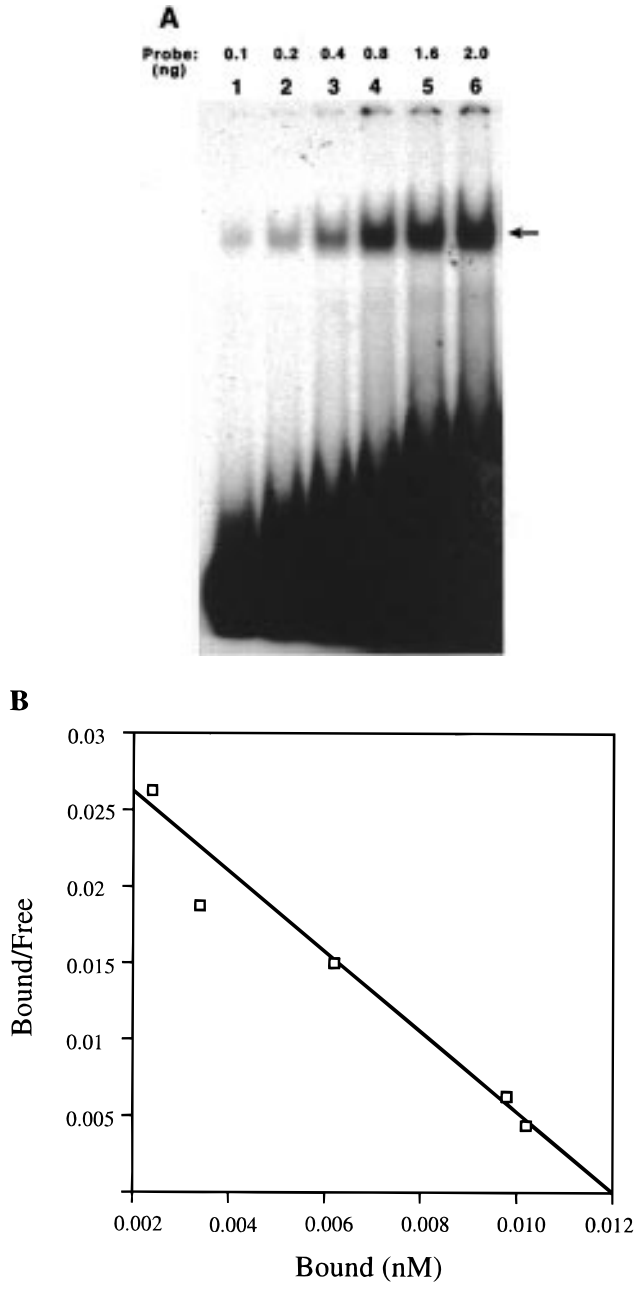

FIGURE 6: Binding affinity of TR2-11-f to the DR4 sequence. (A) Binding of *in vitro* expressed TR2-11-f to the indicated amounts of DR4 probes in the gel-retardation assay. Constant amounts of TR2-11-f were incubated with various amounts of the probes (0.1−2.0 ng) as indicated for each reaction. The arrows indicate the specifically retarded bands. (B) Scatchard plot analysis. The intensity of each retarded band was quantitated using an Imaging Densitometer (Bio-Rad, Model GS-700). The ratio between specific DNA−protein binding (Bound, nM) and free DNA probes (Free, nM) was plotted against the value of specific DNA−protein binding (Bound, nM). The dissociation constant (K_d) and B_{max} were determined as 2.6 nM and 0.012 nM, respectively.

complexes. In addition, by comparing the specific lacZ activities of the positive control and of pBD-TR2/pAD-TR2, the interaction between the ligand-binding domain of TR2-11-f appeared to be at least 10 times stronger than interaction between p53 and SV40.

DISCUSSION

We have characterized negative regulatory effects of TR2-11-f on a DR4 derived from the mouse CRABP-I gene promoter. The biological activities of the two TR2-11 isoforms on this DR4 were demonstrated in cotransfection experiments. TR2-11-f suppressed reporters controlled by this DR4 in the context of either natural or heterologous promoters, whereas TR2-11-t had no significant effects on this DR4. Consistent with this result, only TR2-11-f, but not TR2-11-t, was able to bind to the DR4 sequence (TGACCTTTGGGGACCT), as demonstrated in gel-retardation experiments. TR2-11-f preferentially formed dimeric proteins on this DR4 fragment, as evidenced by the results of gel-retardation experiments using the entire DR4 or a half-site of the DR4 as the probes. The specificity of the TR2-11-f interaction with this DR4 was further confirmed by two experiments, an antibody-mediated supershift experiment and a competition experiment using cold DNA fragments in gel retardation. From mutational analysis of the specific DR4 sequence, it was also concluded that both repeated sites of this DR4 were crucial for this protein−DNA interaction. In addition, the K_d and the B_{max} of this interaction were determined to be 2.6 nM and 0.012 nM, respectively. Finally, using a yeast two-hybrid system, it was shown that the ligand binding domain of TR2-11-f was involved in protein−protein interactions for dimerization. The truncated receptor lacked this domain, and thus could not form dimers and failed to bind to this DR4 sequence.

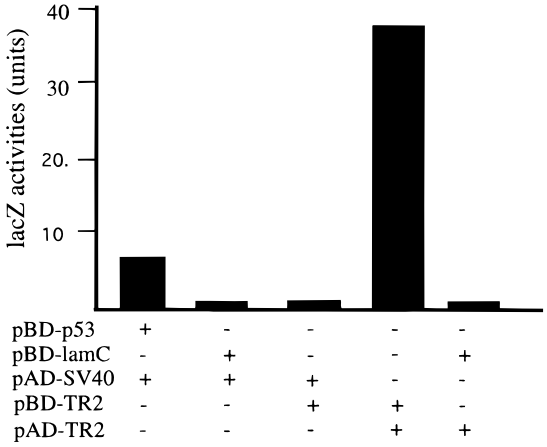

FIGURE 7: Protein−protein interaction demonstrated by the yeast two-hybrid system. The baits and preys were prepared as described under Experimental Procedures. Yeast YRG-2, containing a lacZ reporter controlled by GAL4-binding sites, was cotransfected with the bait and prey pairs as indicated, and plated on the appropriate selection medium. Single colonies were isolated and grown in overnight cultures in selection medium (A_{600} < 1.0), diluted to 0.2 unit of A_{600}, and followed by continuous growth in complete medium until the A_{600} reaches 0.8 unit. A 1.5 mL volume of each culture was collected, concentrated, and lysed by quick-freezing in liquid nitrogen. LacZ activity was determined as described under Experimental Procedures.

The DR4 present in the mouse CRABP-I promoter mediated a strongly suppressive effect on its natural promoter and other heterologous promoters in P19 cells (ref *10* and this study). We have previously identified the thyroid hormone receptor $\beta 1$ ($T_3R_{\beta 1}$) as one potent trans-repressor for this DR4, particularly when it heterodimerized with the retinoid X receptor-β (RXR_β) on the DR4 (*25*). The effects of $T_3R_{\beta 1}/RXR_\beta$ on this DR4 were more physiologically related to CRABP-I gene expression in developing central nervous system in which thyroid hormones and their receptors play a critical role (*29, 30*). In the developing testis, CRABP-I expression was expressed in premeiotic germ cells

such as gonocytes and spermatogonia but was suppressed in the advanced germ cell populations (*17*). As no thyroid hormone receptor expression was detected in these germ cell populations (*17*), the suppression of the CRABP-I gene could not be mediated via the thyroid hormone receptor pathway in the developing germ cell system. Because TR2-11-f expression was specifically elevated in germ cells when meiosis began to take off (*8, 9*), its inhibitory effects on CRABP-I gene expression were physiologically relevant to CRABP-I gene regulation in the testis system. In this study, we provided evidence for a regulatory function of the TR2-11-f receptor on the CRABP-I promoter by binding to the DR4. The regulation by TR2-11-f added an additional player to the already complicated network of regulatory pathways for CRABP-I gene expression (*22*−*25*). Although the function of CRABP-I in germ cell development remained speculative, in vitro studies suggested that CRABP-I could be responsible for shuttling RA inside the cells and/or facilitating RA metabolism into more polar metabolites (*11*−*13*). Numerous studies have demonstrated that a disturbance in vitamin A status resulted in failure of germ cell development in male animals, mostly arrested at premeiotic stages (*31*−*33*). The fact that regulation of the CRABP-I gene was tightly controlled and both the protein sequences and their gene promoters were extremely conserved among animal species suggested a specific role of this protein in important biological processes. From the expression patterns of these two genes in the testis, the most relevant biological process correlated with this regulatory event might be meiosis or post-meiotic differentiation.

Numerous orphan nuclear receptors have been cloned. The identification of their target genes remains the major task to shed light on their physiological roles. TR2-11-f has been found to inhibit RA induction of a DR5-controlled reporter gene, whereas TR2-11-t exerted no consistent effects on this reporters in our previous studies (*8*). The binding of TR2-11-f on several artificial and natural DR sequences was also observed in another study (*34*). From these results, it has been proposed that the TR2-11 family may modulate RA signaling pathways by competition with the RARs or RXRs. However, from the spectrum of TR2-11 expression in animal tissues, the most physiologically relevant organ system for this nuclear receptor action is the reproductive system, particularly during germ cell maturation. Previously, the SV40 late promoter (*35*) and the erythropoietin gene (*36*) promoter have been proposed as the natural targets for the TR2-11 receptor. In this study, the regulation of CRABP-I by TR2-11-f suggested a scenario of physiological significance, in which this orphan nuclear receptor might modulate vitamin A signaling by regulating its metabolic machinery during the process of germ cell maturation that required a homeostatic control of vitamin A supply. Because gene regulation in early germ cells is not totally clear and the regulation of the CRABP-I gene can potentially involve many factors, including intrinsic and hormonal, it remains to be determined if TR2-11-f can interact with other nuclear factors such as corepressors and mediate the suppression of this gene.

Based upon the isolated cDNAs (*6, 8*) and the genomic structure (*7*), several TR2-11 isoforms can be generated. Two isoform receptors designated as TR2-11-f and TR2-11-t are best characterized (*8, 9*). These two receptors are identical in their N-terminal sequence and the DNA-binding domain. Presumably, both isoforms will be able to bind to the same DNA sequences if the ligand-binding domain does not contribute to receptor interaction with DNA. Our studies provided direct evidence for a role of the ligand-binding domain of TR2-11-f in receptor dimerization and a preferred homodimer binding TR2-11-f to this DR4. Consistently, TR2-11-t was not able to suppress reporter containing this DR4 nor could it bind to this DR4. Nevertheless, both the truncated and the full-length receptors were slightly suppressive to the CRABP-I minimal promoter lacking the DR4 (−993 reporter in Figure 1). This could be due to their interaction with certain components involved in the basal transcription machinery. Recent studies have shown that nuclear receptors, such as thyroid hormone receptors, RARs, and RXRs, are able to modulate transcription by multiple mechanisms such as direct binding to the cognate DNA sequences and interacting with certain components of transcription complex (*1, 2*). It is possible that TR2-11 receptors also exert their biological activities by interacting with different transcription factors. The identification of proteins that interact with TR2-11 will provide more evidence for the physiological role of this orphan nuclear receptor family.

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