Characterization of an Inverted Repeat with a Zero Spacer (IR0)-Type Retinoic Acid Response Element from the Mouse Nuclear Orphan Receptor *TR2-11* Gene[†]

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ABSTRACT: An inverted repeat with zero nucleotides in the spacer (IR0, 5′-**GGGTCA CGAACT**-3′) element was localized in the proximal promoter region of the mouse *TR2-11* gene, and characterized as a functional retinoic acid response element (RARE). In gel mobility shift assays, heterodimers of retinoic acid receptor α (RAR_α) and retinoid X receptor β (RXR_β) bound specifically to this element. Neither receptor alone was able to bind to this element efficiently. The dissociation constant (K_d) with respect to RAR−RXR binding was estimated to be 8 nM. The biological activity of this IR0 element was assessed in a heterologous reporter system. The IR0-containing reporter was induced by RA in COS-1 cells in the presence of exogenously provided RAR_α and RXR_β. In addition, the IR0 oligomers could be bound by nuclear extracts isolated from COS-1 cells harboring the expression vectors for RAR_α and RXR_β, but not by extracts isolated from control COS-1 cells. RA responsiveness of this IR0 was further confirmed in P19 cells that expressed endogenous RARs and RXRs. Collectively, these data demonstrated, for the first time, the presence of a natural RARE of the IR0 type, and suggested a potential cross-talk between nuclear orphan receptor TR2-11 and RAR−RXR families.

Nuclear receptors comprise a superfamily of transcription factors, including receptors for steroid hormones (e.g., androgen, estrogen, and glucocorticoid) and nonsteroid hormones (e.g., retinoids, vitamin D, and thyroids) (*1*, *2*). It is believed that upon binding to their lipophilic ligands, nuclear receptors can regulate the activities of their target genes through direct interaction with the cis-acting DNA element, which often consists of two half-sites of the core sequence, AGGTCA (*3*, *4*). Recently, a large number of putative receptors were cloned using low-stringency homology screening, with no ligands identified, named orphan receptors (*5*). Several potential ligands have been identified for some of the orphan receptors. These novel signaling pathways include the biosensor receptor, steroid and xenobiotic-sensing nuclear receptor (SXR) (*6*), and oxysteroid receptor LXR (*7*). In contrast, it has also been shown that some orphan receptors encode constitutive regulatory activities for transcription in transient transfection assays, and they are categorized as activators such as HNF-4 and CAR, and repressors such as TR2, TR4, and COUP-TFs (*5*).

A mouse orphan receptor was isolated and characterized in this lab (*8*), which appeared to be the mouse homologue of full-length human TR2 orphan receptor, TR2-11 (*9*). The expression of this gene was most abundant in the adult mouse testes, particularly in advanced germ cells (*10*), and was affected by vitamin A homeostasis. Vitamin A depletion resulted in a drastic decrease in the level of the *TR2-11* transcript, suggesting a regulatory role of retinoic acid (RA)[1] in the expression of this gene (*10*, *11*). We and others have shown that homodimeric TR2 repressed promoter activities through several potential direct repeat-type response elements (*12*−*15*). Interestingly, unlike most nuclear receptors whose repression activities are attributed to the recruitment of corepressor SMRT/N-CoR through their hinge region (*16*, *17*), the repression domain of TR2 is localized in the very C-terminus of this molecule. This C-terminal region is also responsible for the interaction with its corepressor, RIP140 (*18*). Another unique feature of this receptor is its ability to heterodimerize with orphan receptor TR4 (*19*), but not the common partner retinoid X receptor (RXR) (*5*). These observations suggest that novel ligand-independent actions of orphan receptors may be physiologically relevant.

In an attempt to understand the regulatory mechanisms underlying the testis-specific expression of the *TR2* gene and the potential role of RA in modulating its expression, we have recently identified a 3 kb 5′ upstream sequence of the mouse *TR2* gene and characterized the activities of this regulatory region (manuscript in preparation). Within the defined basal promoter, a highly conserved region containing an inverted repeat with zero nucleotides in the spacer (IR0) was found. To assess the biological function of this sequence, we carried out this study to dissect this element and characterize its biological activities and receptor binding properties. This element was demonstrated to be a functional RARE in reporter assays in COS-1 cells supplemented with

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[1] Abbreviations: IR0, inverted repeat with a zero-nucleotide spacer; RA, retinoic acid; RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, retinoid receptor X; DR, direct repeat; lacZ, β-galactosidase; luc, luciferase.

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RAR_α and RXR_β, and could be bound specifically by RAR_α−RXR_β heterodimers with a high affinity in gel shift assays. Finally, the function of this IR0 element was also examined in P19 cells that expressed endogenous RARs and RXRs. It was suggested that this IR0 element could play an important role in the cross-talk between TR2-11 and RA receptor signaling pathways.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assay and Scatchard Analysis. The mobility shift assay was conducted as described previously (*19*). Briefly, in vitro translated RA receptors were incubated with 1 ng of probe in 20 μL of binding buffer. The probes were prepared by annealing oligonucleotides and labeling the fragments with [α-^{32}P]dCTP using the Klenow enzyme. For competition experiments, 10 and 50 ng of unlabeled oligomers were included in the reaction mixture. The sequences used for gel shift are depicted in Figure 2A. The P1 (sequence shown in Construction of Reporter Genes and Expression Vectors), P2, and P3 oligos were designed to be the same length with different residues mutated in P2 and P3. The dissected sequences, P4 and P5, are also depicted in Figure 2A. For antibody-mediated supershift assays, 1 μL of a 1:100 dilution of RARα monoclonal antibody (Affinity BioReagents, Neshanic Station, NJ) was included in the reaction mixture.

To determine the dissociation constant (K_d), increasing amounts of labeled fragments containing this IR0 (primer P4), ranging from 0.0625 to 4 ng in a reaction solution of 20 μL, were incubated with a fixed amount of receptor proteins. The intensities of the bound and the free probes were determined using a Phosphorimager. The bound:free ratio and the bound value were used for the Scatchard analysis. As a control, similar experiments were also performed for the RAR−RXR binding to the DR5 element derived from *RARβ* promoter (sequence shown in Construction of Reporter Genes and Expression Vectors).

Gel shift assays using nuclear extracts were conducted similarly. In Figures 5 and 6, oligomer P4 was used in the "wild-type" experiments while P5 was used in "mutant". Nuclear extract isolation was performed as described previously (*20*). The extract from a 10 cm dish of cells was resuspended in a volume of 60 μL, and 4 μL (about 10 μg of nuclear protein) was used in each gel shift reaction.

Construction of Reporter Genes and Expression Vectors. The heterologous reporter, IR0-tk-luc, was constructed by inserting the annealed oligomers (P1, 5′-GGATCCAAGCCGAGGGTGGGGTCACGAACTCTGACCCCCATCCCCAAAACACAAACTCGAG-3′) at the *Bam*HI site upstream of a thymidine kinase promoter that directed a luciferase reporter (tk-luc). The mIR0-tk-luc was similarly constructed, with a mutant primer, P2, derived oligomer inserted into the same tk-luc reporter. The DR5-tk-luc (DR5 sequence 5′-AAGGGTTCACCGAAAGTTCACTC-3′) was kindly provided by Dr. R. Evans (*21*). The cDNAs for RAR_α and RXR_β were each cloned into the pSG5 vector at the *Bgl*II site for expression in mammalian cells and in vitro transcription−translation reactions (for gel mobility shift assay).

Cell Culture and Transfection Techniques. The P19 cell line, maintained as described previously (*22*), was used to determine the effects of endogenous RA receptors on the reporter gene activity. COS-1 cells were used in cotransfection experiments to determine the effects of exogenously added nuclear receptors on the reporter gene activity as described previously (*18*). All-trans RA (at-RA) was used in all the RA-treated cell culture experiments. Transfection was conducted by using the calcium phosphate precipitation method. LacZ and luciferase activities were determined as described previously (*18*). The relative luciferase activity has been normalized to the activity of the internal control. Triplicate cultures were used in each transfection experiment, and three independent experiments were conducted to obtain the means and standard errors (SEM) for all the transfection experiments.

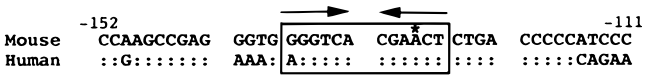

FIGURE 1: Sequence comparison of a conserved IR0 element from the proximal promoter region of the *TR2-11* gene. Conserved sequences are denoted with colons. The sequences of the IR0 element are boxed and labeled with a pair of arrows. The numbers (relative to the translation starting site) indicating the position of this element correspond to the mouse sequence (Genbank accession number U96095). The shortest 5′ cDNA end is labeled with an asterisk. The position of human IR0 is −77 to −88 relative to the major transcription initiation site (*23*).

RESULTS

Characterization of the IR0 Element. Our previous studies showed a dramatic decrease in the level of *TR2-11* expression in the testes of vitamin A deficient mice (*10*), suggesting a regulatory effect of RA on the expression of this gene. Recently, we have characterized the 5′ regulatory region of the mouse *TR2-11* gene and observed RA induction of this gene in P19 cells (manuscript in preparation). By aligning the upstream sequence of the mouse gene with that of the human's (*23*), we identified a highly conserved IR0 element in the proximal promoter region, which could potentially function as a retinoic acid response element (RARE) (Figure 1). Interestingly, the position of this element is adjacent to the shortest 5′ cDNA end of the mouse *TR2-11* identified via 5′ rapid amplification of the cDNA end (5′ RACE) experiment (Figure 1), while the position in the human gene is 77 bp upstream of the transcriptional initiation site (*23*). To determine whether this IR0 could be a response element for RAR and/or RXR, gel mobility shift assays were first carried out using a DNA fragment containing this IR0 and its flanking sequences. Radioactive DNA oligomers containing this conserved sequence were incubated with in vitro translated nuclear receptor proteins, separated by polyacrylamide gel electrophoresis, and examined by autoradiography. As shown in Figure 2, RAR alone could not shift this element (lane 1) and RXR alone exhibited only negligible binding (lane 2). In contrast, addition of RAR and RXR together resulted in obvious retardation of this fragment (lane 3), indicating a strong binding of this fragment by heterodimeric receptors. Furthermore, the specific protein−DNA complexes could be supershifted by the addition of an antibody against RAR (lane 4).

To further dissect the sequence responsible for RAR−RXR binding, mutation and deletion analyses of this sequence were conducted. Mutation at the sequence corresponding to the 5′ half-site of IR0 resulted in a dramatic decrease in the level

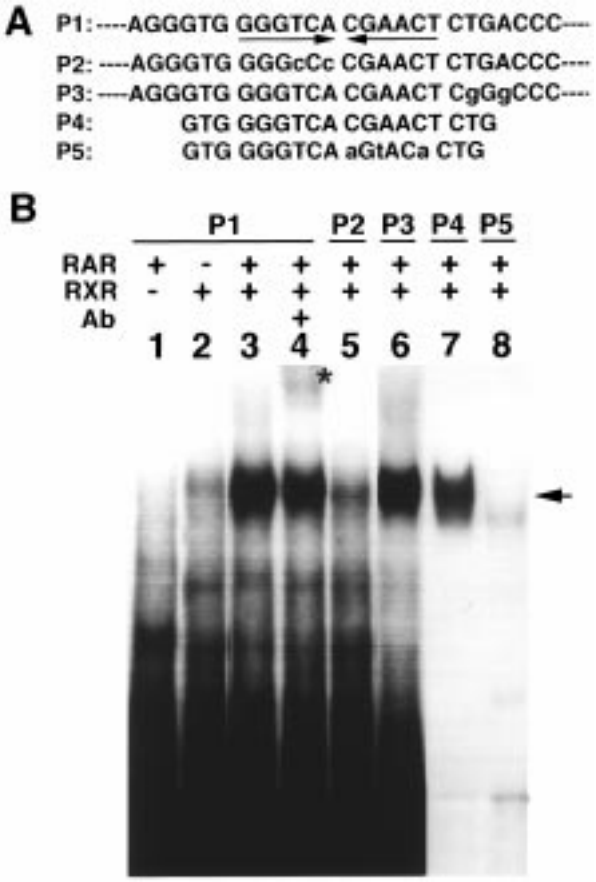

FIGURE 2: $RAR_{\alpha}-RXR_{\beta}$ heterodimers binding to the IR0 element. (A) Sequences of the IR0 and its mutants used in the gel mobility shift assay. The putative IR0 is labeled with a pair of arrows. The mutated residues are shown in lowercase. The complete sequence of P1−P3 is described in Materials and Methods. (B) Gel mobility shift assay showing binding of RAR−RXR heterodimers to IR0. The upper panel shows the receptor protein, antibody, and the labeled oligomers used in DNA binding reactions. The retarded protein−DNA band is denoted with an arrow on the right, and the supershifted band mediated by an anti-RAR antibody is denoted with an asterisk.

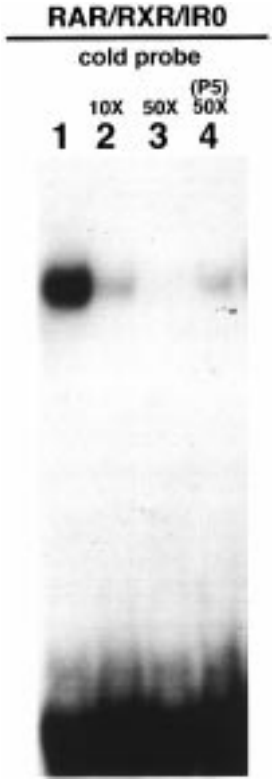

FIGURE 3: Specific binding of RAR−RXR to the IR0 element. Oligomer P4 (see Figure 2) was used in the competition experiments. Lanes 2 and 3 contained reactions competed with a 10- and 50-fold excess of nonlabeled DNA fragments, respectively. In lane 4, oligomer P5 was used for competition.

of receptor DNA binding (lanes 5). In contrast, mutation at its flanking sequence had no effect on receptor binding (lane 6). Consistently, RAR−RXR binding was not affected by deleting most of its flanking sequences (lane 7). Moreover, the majority of nonspecific binding observed in the assays using the long probe (lanes 1−6) was abolished when using the smaller probe containing only the IR0 and three nucleotides of each flanking region (lane 7). As expected, mutating the 3′ half-site of IR0 also resulted in a drastically decreased level of RAR−RXR binding (lane 8). These results show that the IR0 located in the proximal promoter region of *TR2-11* gene can be bound by RAR−RXR heterodimers.

Specific and High-Affinity Binding of RAR−RXR to the IR0 Element. The data shown above suggested that IR0 could be a target site for RAR−RXR heterodimers. We then performed competition experiments to confirm the specificity of receptor binding to this element. Since oligomer P4 exhibited the most specific binding pattern with RAR−RXR (Figure 2, lane 7), this sequence was then used in this cold competition and the following gel shift experiments. As shown in Figure 3, the RAR−RXR-retarded band was competed out, in a dose-dependent manner, by the addition of unlabeled DNA fragments (10- and 50-fold excess in lanes 2 and 3, respectively). However, the oligomer with the 3′ half-site mutated (P5), even when present in a 50-fold excess, was not able to efficiently compete for receptor binding (lane 4). In fact, a 10-fold excess of the wild-type oligomer was able to compete as efficiently as a 50-fold excess of the mutated oligomer (comparing lanes 2 and 4) for binding.

Scatchard analyses were performed to estimate the binding affinity of RAR−RXR for this dissected IR0 element. Increasing amounts of labeled IR0 DNA fragments (fragment P4, from 0.0625 to 4 ng in a total reaction volume of 20 μL) were incubated with a fixed amount of receptor proteins, and the reactions were resolved on an acrylamide gel as shown in Figure 4A. The intensities of the bound and the free probes were quantified using a Phosphorimager. Figure 4D shows the results of a Scatchard plot analysis. The calculated dissociation constant (K_d) for RAR−RXR binding to this element was approximately 8 nM, which was comparable to the K_d of RAR−RXR binding to the DR5 derived from the *RARβ* promoter (about 2 nM) (ref *13* and Figure 4C). Consistent with the result depicted in Figure 2, the K_d for RXR homodimer binding to this element could not be determined, since its binding to IR0 did not reach saturation at the highest concentration of oligomers, suggesting that RXR binds to IR0 with a very low affinity (Figure 4B). Collectively, these data show a specific and high-affinity binding of RAR−RXR to the IR0 element.

Biological Activity of the IR0 Element. To determine whether the IR0 was a functional RARE in the context of a heterologous promoter, the dissected IR0 was placed upstream of a tk-luciferase reporter, designated as IR0-tk-luc. For a control, a reporter carrying the mutated IR0, which

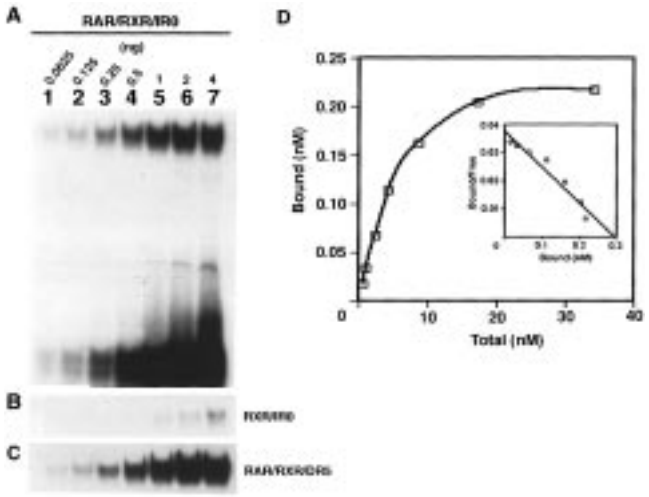

FIGURE 4: High-affinity binding of the $RAR_\alpha-RXR_\beta$ heterodimer to the IR0 element. (A) Gel mobility shift assay with a fixed amount of RAR−RXR receptor proteins incubated with increasing amounts of labeled probe as indicated. (B and C) Negative and positive controls, respectively, showing low-affinity binding of the RXR homodimer to IR0 and high-affinity binding of RAR−RXR to the DR5 element (sequence in Materials and Methods). (D) Scatchard analysis for RAR−RXR binding to IR0. The intensities of the specifically retarded band (bound) and the band representing the free probes in panel A were quantified using a Phosphorimager. The resulting Scatchard plot is shown. The calculated K_d was approximately 8 nM.

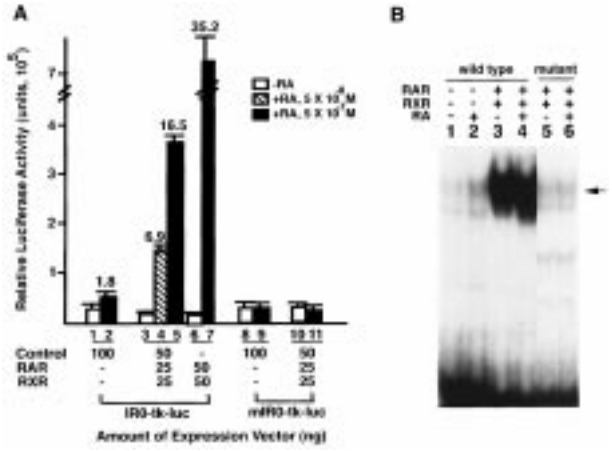

FIGURE 5: Effects of RAR and RXR on RA induction of reporter activities in COS-1 cells. (A) RA induction of heterologous reporters containing IR0 from the *TR2-11* gene promoter. The wild-type and mutant IR0 fragments were each placed at the 5′ end of a tk-luc reporter. Relative luciferase activities were determined by normalizing the luciferase activity to the lacZ activity (for transfection control). White bars depict results from experiments without RA, and hatched and black bars depict results from experiments with RA at different concentrations. The doses of the RAR−RXR expression vectors are listed in the lower panel. The fold induction was calculated by comparing the relative luciferase activity of RA-treated cultures to that of control (no RA), and is indicated above each corresponding bar. (B) Gel mobility shift experiment using nuclear proteins from COS-1 cells. Nuclear proteins isolated from COS-1 cells transfected with either control or a mixture of RAR and RXR expression vectors were examined for DNA binding. The upper panel shows the expression vectors transfected in the cells and the oligomer used (wild-type or mutant IR0). RA was added in the culture at a final concentration of 5×10^{-7} M as indicated. The retarded DNA band is denoted with an arrow.

cannot be bound efficiently by RAR−RXR (Figure 2B), was also generated, designated as mIR0-tk-luc. COS-1 cells were cotransfected with either one of the two reporters, a control sv40-lacZ vector, together with either the control, or the RAR and RXR expression vectors. As shown in Figure 5A, addition of RAR and RXR expression vectors dramatically increased the reporter activity in a ligand concentration (columns 3−5)- and receptor dose (columns 5 and 7)-dependent manner. An induction of more than 35-fold was observed when RAR and RXR expression vectors were each added in the amount of 50 ng (column 7). The wild-type IR0-tk-luc was induced by RA for about 2-fold in the absence the exogenously added RAR−RXR (columns 1 and 2), possibly due to the presence of some endogenous RARs−RXRs in COS-1 cells. As excepted, the reporter containing the mutated IR0 was not induced by RA (columns 8−11).

To examine whether RA induction of the reporter activity in COS-1 cells was associated with the exogenously provided RAR−RXR, gel shift experiments were conducted to compare shifted patterns of IR0 by nuclear extracts isolated from control COS-1 cells and cells transfected with RAR−RXR expression vectors. As shown in Figure 5B, nuclear extracts from cells transfected with the control (empty) vectors had little DNA binding activity (lanes 1 and 2). However, nuclear extract isolated from cells transfected with RAR−RXR expression vectors exhibited an intense DNA binding signal (lane 3). Addition of RA only slightly increased the amount of receptors binding to the IR0 element (lane 4). Similar results were obtained when the DR5 element was used (data not shown). On the other hand, the extent of binding of nuclear extract from RAR−RXR transfecting cells to the mutated IR0 decreased dramatically (lanes 5 and 6). This observation was consistent with the transfection data in that the activity of the reporter containing the mutated IR0 elements could not be induced by addition of RA and RA receptors (Figure 5A, lanes 10 and 11). These results suggest that the IR0 derived from the mouse *TR2-11* promoter is a functional RARE, and the induction of IR0-containing reporter activity is mediated by ligand-bound RAR−RXR heterodimers.

IR0-Mediated RA Induction of Reporter Activity in P19 Cells. RA has been shown to induce differentiation and apoptosis in P19 embryonal carcinoma cells (*24*−*27*). The expression of *RARβ* is induced by RA in P19 cells through a DR5-type RARE (*21*, *28*) found in the proximal promoter region of this gene. To assess the function of this IR0 in a physiological situation, i.e., to ask whether this IR0 is responsive to RA induction in cells that normally respond to RA induction and contain reasonable amounts of endogenous retinoid receptors, P19 cells were used as a model system. Cells were transfected with either the IR0-tk-luc (wild-type reporter) or the mIR0-tk-luc (mutant reporter), together with a lacZ internal control vector, without adding exogenous RAR−RXR. A luciferase reporter containing the DR5 derived from the *RARβ* gene was included in the transfection assay as a positive control for RA response. Following the addition of RA, cells were harvested at 12 and 24 h and the reporter activities were determined. As shown in Figure 6, the activities of both IR0-tk-luc (columns 1−4) and DR5-tk-luc (columns 9−12) were induced. Consistent with the results obtained from COS-1 studies, RA failed to induce the reporter containing the mutant IR0 (mIR0-tk-luc) (columns 5−8). In a parallel experiment,

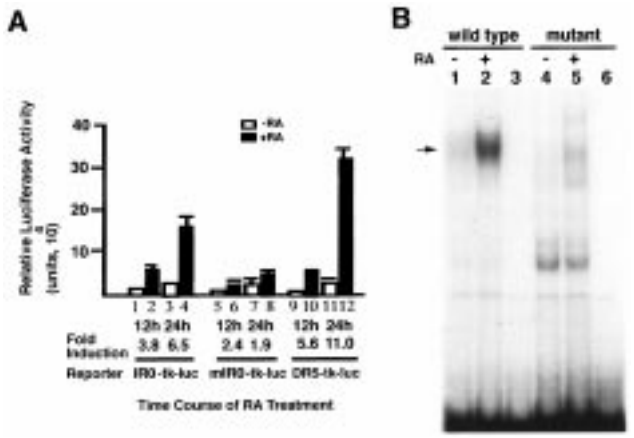

FIGURE 6: Activation of IR0-containing reporter by endogenous RARs−RXRs in P19 cells. (A) The RA inducibility of heterologous promoters containing a wild-type IR0, a mutant IR0 (see Figure 5A), or a DR5 derived from the *RARβ* gene were determined in P19 cells without introducing exogenous retinoid receptors. White and black bars represent data from cells without and with RA treatment (5×10^{-7} M), respectively. RA induction of reporter activity was determined 12 and 24 h after drug administration. (B) Gel mobility shift experiment using nuclear proteins from P19 cells. The upper panel shows the oligomer used (wild-type or mutant IR0). RA was added to the culture at a final concentration of 5×10^{-7} M as indicated. Lanes 3 and 6 contained free probes of the wild-type and mutant oligomers, respectively. The retarded band is denoted with an arrow.

nuclear extracts of P19 cells were isolated 24 h after RA or vehicle treatment so their DNA binding activities could be examined in a gel shift experiment. A drastically increased IR0 binding activity was observed for the RA-treated nuclear extracts (Figure 6B, compare lane 1 to 2), and this DNA binding activity was almost completely abolished when the mutated element was used (lanes 4 and 5). These results correlated well with the RA responsiveness of reporter genes in the transient transfection assays. These data show that IR0 is also a functional RARE in the context of P19 cell physiology.

DISCUSSION

An IR0 element was found in both the mouse and the human *TR2-11* gene promoters. Gel shift assays demonstrated the specific binding of this element by RAR−RXR heterodimers, as well as the requirement of both half-sites of this IR0 element in retinoid receptor binding. Scatchard analysis revealed that RAR−RXR bound specifically to IR0 with a high affinity (estimated K_d of 8 nM). The activity of a heterologous promoter containing the wild-type IR0 element, but not the mutated element, was induced by RA in the presence of exogenous RA receptors in COS-1 cells or endogenous RA receptors in P19 cells. Consistent with these results, only the wild-type IR0 element can be bound by RA receptor-containing nuclear extract from either cell line, suggesting that IR0 is a functional RARE in vivo, and the induction of reporter activity by RA is mediated through direct RAR−RXR binding to the IR0 element.

The conserved (Pu)G(G/T)TCA half-site sequence is found in most of the RAREs that have been identified. Most of the reported RAREs are in a head-to-tail (direct repeat) orientation such as DR5 from *RARβ* and DR1 from *CRBP II* (*29*). Interestingly, the two half-sites located in the *TR2-11* minimal promoter are arranged as an imperfect palindromic IR0 (**GGGTCA CGAACT**). A recent sequence comparison study reveals that a third half-site is present 3′ to this IR0, with a potential to form an "IR7" (**GGGTCA** CGAACTC **TGACCC**; see Figure 1) (*30*). However, no experimental data were provided in this study. In our studies presented here, the goal was to examine the biochemical properties and biological activities of this IR0 by using reporter and gel shift assays, respectively. By making point mutations and deletion mutations, we have demonstrated that the sequence corresponding to the 3′ half-site of the IR7 is not required for RAR−RXR binding (Figure 2), whereas both half-sites of the IR0 are essential for receptor binding and RA responsiveness (Figures 2, 5, and 6). These data clearly provide the evidence for the function of IR0, but not IR7, with respect to RA response. It is known that the arrangement of two half-sites of RARE is more flexible than that of the response element for steroid hormone receptors. While most of the natural RAREs consisted of a pair of direct repeats with a spacer ranging from one to five nucleotides (*29*), several atypical response elements have been identified recently, such as an IR6 from chicken lysozyme gene and myelin basic protein gene (*31*) and an everted repeat (ER) 8 from the *γ* F-crystalline gene (*32*). For IR0, it has only been demonstrated by using a synthetic DNA fragment (*29*, *33*). Our identification of the first natural IR0 derived from the *TR2-11* gene adds a new category to the polymorphism of the natural RAREs.

In our other study, we have characterized the 3 kb regulatory region of the mouse *TR2-11* gene and defined the basal promoter activity (manuscript in preparation). In this GC rich, proximal region, no TATA box was found. By using 5′ RACE approaches, we have identified multiple transcription initiation sites of the mouse *TR2-11* gene. Intriguingly, the shortest 5′ cDNA end found is located within the IR0 element (Figure 1). However, the location of IR0 in the human gene is 77 bp upstream from the mapped major initiation site, a position similar to that of the DR5 RARE in the *RARβ* gene proximal promoter (*34*). This IR0 element appears to be the only potential RARE in the 3 kb upstream sequence of both mouse and human species by computer analyses (*23*). From promoter deletion studies, our previous results also showed that sequences of the *TR2-11* basal promoter (212 bp upstream from the translation starting site and 83 bp upstream from the shortest 5′ cDNA end), which contained the IR0, were sufficient for the activation by ligand-bound RAR−RXR (manuscript in preparation). While the precise position of IR0 relative to the transcription initiation site could not be defined because of the presence of multiple transcription initiation sites in this promoter, this element must be close to the binding site for the basal transcriptional machinery so that RAR−RXR can physically interact with the components of transcription machinery, as shown in another study (*35*).

Although RA can induce the IR0-containing reporter activity in RAR−RXR transfected COS-1 cells, it did not significantly increase the DNA binding activity of RAR−RXR to the IR0 element (Figure 5, lanes 3 and 4). This observation agrees with the current model for ligand-dependent transactivation and ligand-independent transrepression by nuclear receptors (*16*, *17*, *36*−*38*), in which the ligand binding-induced conformational change facilitates the

release of corepressors from, and recruitment of coactivators to, receptors. The RA-induced DNA binding activity of P19 nuclear extract (Figure 6, lanes 1 and 2) is likely due to the increased level of RA receptor expression, such as the RARβ gene (*21*, *28*, *34*), as a result of RA treatment. Our recent data have indicated that the endogenous *TR2-11* mRNA level is moderately induced by RA treatment as compared to that of the RARβ gene in P19 cells (manuscript in preparation). Consistent with this observation, the induction level of IR0 containing reporter activity (IR0-tk-luc, 6.5-fold) is weaker than that of DR5 containing reporter activity (DR5-tk-luc, 11-fold) in P19 cells. With a highly competitive K_d of this IR0 (as compared to DR5) for RAR−RXR binding, it is tempting to speculate that other nuclear factors in P19 cells may be involved in modulating the effects of RA on this IR0. It is possible that coregulators that interact with holo-RAR−RXR occupying this IR0 can be involved in the manifestation of RA effects on this element. In the future, it would be interesting to address how coactivators may differentially interact with RAR−RXR with respect to binding to the IR0 and other response elements such as DR5.

The physiological roles of several orphan receptors have been revealed in gene-knockout mice (*2*). Studies in which various biological systems have been employed suggest that the biological functions of TR2-11 are most relevant to the RA signaling pathways. For instance, TR2-11 regulates several genes that are also RA-target genes by interacting with common response elements (*12*, *13*, *15*). Furthermore, TR2-11 utilizes RIP140 as a corepressor (*18*), while RIP140 is also a coregulator of RAR and ER (*39*, *40*). In this study, we have identified a functional IR0-type RARE from the *TR2-11* promoter. This study extends our current understanding of cross-talk between these two nuclear receptor families, and suggests a potentially important role of TR2-11 in vitamin A action.

REFERENCES

1. Beato, M., Herrlich, P., and Schutz, G. (1995) *Cell 83*, 851−857.
2. Kastner, P., Mark, M., and Chambon, P. (1995) *Cell 83*, 859−869.
3. Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) *Cell 65*, 1255−1266.
4. Glass, C. K. (1994) *Endocr. Rev. 15*, 391−407.
5. Mangelsdorf, D., and Evans, R. M. (1995) *Cell 83*, 841−850.
6. Blumberg, B., Sabbagh, W., Jr., Juguilon, H., Bolado, J., Jr., van Meter, C., Ong, E., and Evans, R. M. (1998) *Genes Dev. 12*, 3195−3205.
7. Janowski, B. A., Willy, P. J., Devi, T. R., Falck, J. R., and Mangelsdorf, D. J. (1996) *Nature 383*, 728−731.
8. Lee, C.-H., Copeland, N. G., Gilbert, D. J., Jenkins, N. A., and Wei, L.-N. (1995) *Genomics 30*, 46−52.
9. Chang, C., Kokontis, J., Acakpo-Satchivi, L., Liao, S., Takeda, H., and Chang, Y. (1989) *Biochem. Biophys. Res. Commun. 165*, 735−741.
10. Lee, C.-H., Chang, L., and Wei, L.-N. (1996) *Mol. Reprod. Dev. 44*, 305−314.
11. Lee, C.-H., Chang, L., and Wei, L.-N. (1997) *J. Endocrinol. 152*, 245−255.
12. Chinpaisal, C., Lee, C.-H., and Wei, L.-N. (1998) *J. Biol. Chem. 273*, 18077−18085.
13. Lin, T.-M., Young, W.-J., and Chang, C. (1995) *J. Biol. Chem. 270*, 30121−30128.
14. Lee, H.-J., and Chang, C. (1995) *J. Biol. Chem. 270*, 5434−5440.
15. Chinpaisal, C., Chang, L., Hu, X., Lee, C.-H., Wen, W.-N., and Wei, L.-N. (1997) *Biochemistry 36*, 14088−14095.
16. Chen, J. D., and Evans, R. M. (1995) *Nature 377*, 454−457.
17. Horlein, A. J., Naar, A. M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamel, Y., Soderstrom, M., Glass, C. K., and Rosenfeld, M. G. (1995) *Nature 377*, 397−403.
18. Lee, C.-H., Chinpaisal, C., and Wei, L.-N. (1998) *Mol. Cell. Biol. 18*, 6745−6755.
19. Lee, C.-H., Chinpaisal, C., and Wei, L.-N. (1998) *J. Biol. Chem. 273*, 25209−25215.
20. Zhang, X., Huang, C. J., Nazarian, R., Ritchie, T., de Vellis, J. S., and Nobel, E. P. (1997) *BioTechniques 22*, 849−850.
21. Sucov, H. M., Murakami, K. K., and Evans, R. M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 5392−5396.
22. Wei, L.-N., Blaner, W. S., Goodman, D. S., and Nguyen-Huu, M. C. (1989) *Mol. Endocrinol. 3*, 454−463.
23. Lin, D. L., Wu, S. Q., and Chang, C. (1998) *Endocrine 8*, 123−134.
24. Bain, G., Ray, W. J., Yao, M., and Gottlieb, D. I. (1994) *BioEssays 16*, 343−348.
25. Horn, V., Minucci, S., Ogryzko, V. V., Adamson, E. D., Howard, B. H., Levin, A. A., and Ozato, K. (1996) *FASEB J. 10*, 1071−1077.
26. Suzuki, T., Kim, H.-S., Kurabayashi, M., Hamada, H., Fujii, H., Aikawa, M., Watanabe, M., Watanabe, N., Sakomura, Y., Yazaki, Y., and Nagai, R. (1996) *Circ. Res. 78*, 395−404.
27. Yao, M., Bain, G., and Gottlieb, D. I. (1995) *J. Neurosci. Res. 41*, 792−804.
28. de The, H., Vivanco-Ruiz, M. M., Tiollais, P., Stunnenberg, H., and Dejean, A. (1990) *Nature 343*, 177−180.
29. Sucov, H. M., and Evans, R. M. (1995) *Mol. Neurobiol. 10*, 169−183.
30. Le Jossic, C., and Michel, D. (1998) *Biochem. Biophys. Res. Commun. 245*, 64−69.
31. William, G. R., Zavacki, A. M., Harney, J. M., and Brent, G. A. (1994) *Endocrinology 134*, 1888−1896.
32. Tini, M., Otulakowske, G., Breitman, M. L., Tsui, L. C., and Giguere, V. (1993) *Genes Dev. 7*, 295−307.
33. Heery, D. M., Pireeat, B., Gronemeyer, H., Chambon, P., and Losson, R. (1994) *Nucleic Acids Res. 22*, 726−731.
34. Hoffmann, B., Lehmann, J. M., Zhang, X.-K., Hermann, T., Husmann, M., Graupner, G., and Pfahi, M. (1990) *Mol. Endocrinol. 4*, 1727−1736.
35. Mengus, G., May, M., Carre, L., Chambon, P., and Davidson, I. (1997) *Genes Dev. 11*, 1381−1395.
36. Horwitz, K. B., Jackson, T. A., Bain, D. L., Richer, J. K., Takimoto, G. S., and Tung, L. (1996) *Mol. Endocrinol. 10*, 1167−1177.
37. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) *Cell 87*, 953−959.
38. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1997) *Nature 389*, 194−198.
39. Cavailles, V., Dauvois, S., L'Horset, F., Lopez, G., Hoare, S., Kushner, P. J., and Parker, M. G. (1995) *EMBO J. 14*, 3741−3751.
40. Joyeux, A., Cavailles, V., Balaguer, P., and Nicolas, J. C. (1997) *Mol. Endocrinol. 11*, 193−202.

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