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Characterization of the Mouse Nuclear Orphan Receptor TR2-*11* Gene Promoter and Its Potential Role in Retinoic Acid-Induced P19 Apoptosis

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ABSTRACT. The complete mouse orphan nuclear receptor *TR2–11* gene structure and its 5′-untranscribed region were characterized. This gene contains 14 exons, with the first exon encoding only the 5′-untranslated sequence. The regulatory region of this gene was characterized by using reporter assays that define the minimal promoter activity in a sequence 212 nucleotides upstream from the translation initiation site. Furthermore, it was concluded that splicing of intron 1 is required for efficient promoter activity. Reporters driven by this promoter were induced by retinoic acid (RA) in COS-1 cells supplied with exogenous retinoic acid receptor-α (RAR_α) and retinoid receptor X-β (RXR_β). Binding of RAR_α/RXR_β to the minimal promoter region was demonstrated in gel retardation assays. In P19 cells, both the endogenous *TR2–11* gene and the reporters driven by this promoter were induced by RA in a protein synthesis-independent manner, and overexpression of TR2–11 protein resulted in cellular apoptosis in the absence of RA. The regulation of TR2–11 by RA and the implication of TR2 up-regulation in P19 cellular apoptosis are discussed. BIOCHEM PHARMACOL **60**;1:127–136, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. apoptosis; orphan receptor; P19 embryonal carcinoma cells; retinoic acid; *TR2–11* gene; TR2–11 expression

Nuclear receptors are transcription factors that regulate gene expression in a wide variety of biological processes, such as growth, differentiation, and development. Interactions of these receptors with the transcription machinery result in activation or suppression of gene expression [1–5]. Recently, a large number of nuclear receptors with no known ligands, named orphan nuclear receptors, were identified; however, the physiological functions of these orphan receptors remain elusive. Nevertheless, by gene-targeting approaches, data have been obtained that strongly support the notion that orphan nuclear receptors play important, sometimes essential roles in animal physiology [6].

The mouse orphan receptor TR2–11 was isolated and characterized in this laboratory [7, 8]. In our previous study, its chromosomal localization was assigned, designated as *TR2–11*, and the entire coding region was found to be encoded by 13 exons [8]. In animal studies, the expression of this gene has been shown to reflect vitamin A status. For instance, this gene is activated during embryonic stages that are vitamin A-sensitive, such as early- to mid-gestation stages [9, 10]. In postnatal stages, this gene is expressed most abundantly in the male gonads, specifically in the advanced germ cells. Additionally, vitamin A depletion results in a drastic decrease of TR2–11 expression in the testes, suggesting a regulatory role of RA† on the expression of this gene. In cultured cells, this gene is expressed weakly in the stem cell population of a mouse embryonal carcinoma cell line, P19 [10].

Because of the high similarity of the DNA binding domain of TR2–11 to that of the retinoid receptor families, RARs and RXRs, the biological function of TR2–11 was first examined in RA signaling pathways. A negative regulatory role of TR2–11 in RA signaling pathways was suggested by using reporters regulated by various RAREs such as a DR5-type RARE derived from the RAR_β gene [11–13], a DR4-type element derived from the mouse cellular retinoic acid binding protein I gene [14], and other dissected DR2 and DR4 elements [15]. In these studies, it was demonstrated that TR2–11 suppressed RA induction of these heterologous reporters in a concentration-dependent manner by competing with RAR/RXR binding to the RARE. Furthermore, in an attempt to identify coregulators of TR2–11, we conducted yeast two-hybrid screening ex-

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† *Abbreviations*: RA, retinoic acid; GFP, green fluorescent protein; IR, inverted repeat; lacZ, β-galactosidase; PCR, polymerase chain reaction; PI, propidium iodide; 5′-RACE, 5′-rapid amplification of cDNA ends; DR, direct repeat; RAR, retinoic acid receptor; RXR, retinoid receptor X; RARE, retinoic acid response element; RIP140, receptor interacting protein 140; RT–PCR, reverse transcription coupled–polymerase chain reaction; and TR2–11, testis receptor 2–11.

periments and showed that TR2–11 utilizes RIP140 as a co-repressor, which was shown to interact with RARs/RXRs in a ligand-dependent manner [16]. In other systems, TR2–11 also has been shown to play a repressive role in reporter expression directed by a DR2- or a DR4-type element [17, 18]. Based upon these studies, it was suggested that TR2–11 could play a modulatory role in RA signaling pathways.

In this study, the regulatory region of the *TR2–11* gene was identified, providing evidence for RA regulation of this gene. Furthermore, the relationship of TR2–11 overexpression and apoptosis in P19 cells was examined, suggesting a role of TR2–11 in an RA-induced, specific cellular event in P19 cultures.

MATERIALS AND METHODS

Cloning, Sequencing, and mRNA Analyses

To obtain the complete 5′-untranslated sequence of the mouse *TR2–11* transcripts, 5′-RACE reactions were conducted as described [19]. Briefly, mouse embryonic mRNA was reverse transcribed using a primer specific to the N-terminal portion of the *TR2–11* coding region (5′-CATCTGTTGGTCGATAATTT-3′, starting at the 50th nucleotide downstream of the translation initiation codon) [8], tailed with poly(C) using terminal transferase, and followed by PCR using the N-terminus-specific primer described above and a 5′ anchor primer containing multiple cloning sites (5′-CTACTACTAGGCCACGCGTC-GACTAGTACGGGGGGGGGGGGGG-3′). The amplified clones contained inserts of approximately 250 bp in length. Poly(A) RNA was isolated from mouse embryos at gestation day 12.5 using the method described by Cathala *et al*. [20]. A mouse genomic DNA library constructed at the *Bam*HI site of the λDASH II vector (Stratagene) was screened using probes prepared from the amplified DNA fragments. Northern blot analysis was conducted as described using the unique 3′-end sequence of the cDNA as the probe [10]. RT–PCRs were performed by using oligo d(T) primers in reverse transcription reactions in a total volume of 20 μL, followed by an amplification program of 94° for 45 sec, 55° for 45 sec, and 72° for 1 min, for a total of 30 cycles. TR2–11-specific, 320-bp fragments, which spanned exon 2 to exon 5, were amplified by using primers 5′-TAGAAGAAATTGCACATC-3′ and 5′-CGACAG-GAATAGACTAG-3′ [8, 9]. A pair of actin-specific primers, 5′-TGGCCTTAGGGTGCAGGG-3′ and 5′-GT GGGCCGCTCTAGGCACCA-3′, were included for 23 cycles in each reaction for internal controls. Under these conditions, both actin and TR2–11 transcripts could be amplified in a linear fashion. Following PCR, 5 μL of each sample was analyzed on Southern blots using probes prepared from the mouse TR2–11 and actin cDNAs. The hybridization signals were quantified by using Image Quant software (Molecular Dynamics). The TR2–11 signal was normalized to the actin signal in each reaction to obtain a value representing the specific TR2–11 expression level. Using the expression level of the control reaction as an arbitrary value of 1, the relative expression level of TR2–11 in each treatment was determined. The means and SD were obtained from three experiments.

Construction of Reporter Genes and Expression Vectors

A genomic fragment containing an approximately 3-kb upstream sequence, exon 1, the entire intron 1, and exon 2 (encoding the translation initiating codon) was used as the starting material to construct all the reporters. An *Escherichia coli lacZ* structural gene isolated from pMCI-1871 (Pharmacia) in a *Bam*HI fragment was inserted, in-frame, at a PCR-generated *Bam*HI site at the fourth codon of the *TR2–11* coding region in the genomic segment, generating the longest construct, TR2-lacZ/in, with the entire intron 1 retained. To engineer TR2-lacZ/del-in, the lacZ cassette was inserted at the same PCR-generated *Bam*HI site of TR2–11 cDNA, which then was fused to the 3-kb 5′ upstream sequence derived from the genomic clone, generating a reporter with the entire intron 1 deleted. To engineer TR2-lacZ, the TR2-lacZ/in was deleted by *Eco*RV and *Sma*I digestion to delete 11 kb of intron 1, with approximately 250 bp of sequence retained at both the splicing donor and acceptor sites. All the subsequent promoter deletion mutants were constructed from the TR2-lacZ vector by digesting the parental vector with restriction enzymes, *Nco*I (−1938), *Bgl*II (−1738), *Hin*dIII (−1228), or *Stu*I (−388), or by fusing small PCR fragments to the 5′-end of the lacZ cassette (for constructs −312, −262, −212, and −163).

The RAR_β-lacZ (originally named Sil-REM/β-gal-Neo) constructs were provided by Dr. M. Wagner [21]. The GFP-TR2 fusion was constructed as described previously [16]. The cDNAs for RAR_α and RXR_β each were cloned into the pSG5 vector at a *Bgl*II site for expression in mammalian cells.

Cell Culture, Transfection, and the Detection of Apoptosis

The P19 cell line, maintained as described previously [22], was used to examine RA regulation of endogenous *TR2–11* gene expression as well as reporter activities. COS-1 cells were used in co-transfection experiments to determine the effects of exogenously added nuclear receptors on the reporter gene activity as described [16]. Transfection was performed by using the calcium phosphate precipitation method. LacZ and luciferase activities were determined as described [13]. The relative reporter activity was determined by normalizing the reporter activity to that of the internal control. Triplicate cultures were used in each transfection experiment, and three independent experiments were conducted to obtain the means and SEM for all the transfection experiments.

To study the effect of TR2–11 overexpression, P19 cells were transfected with the control GFP vector or the

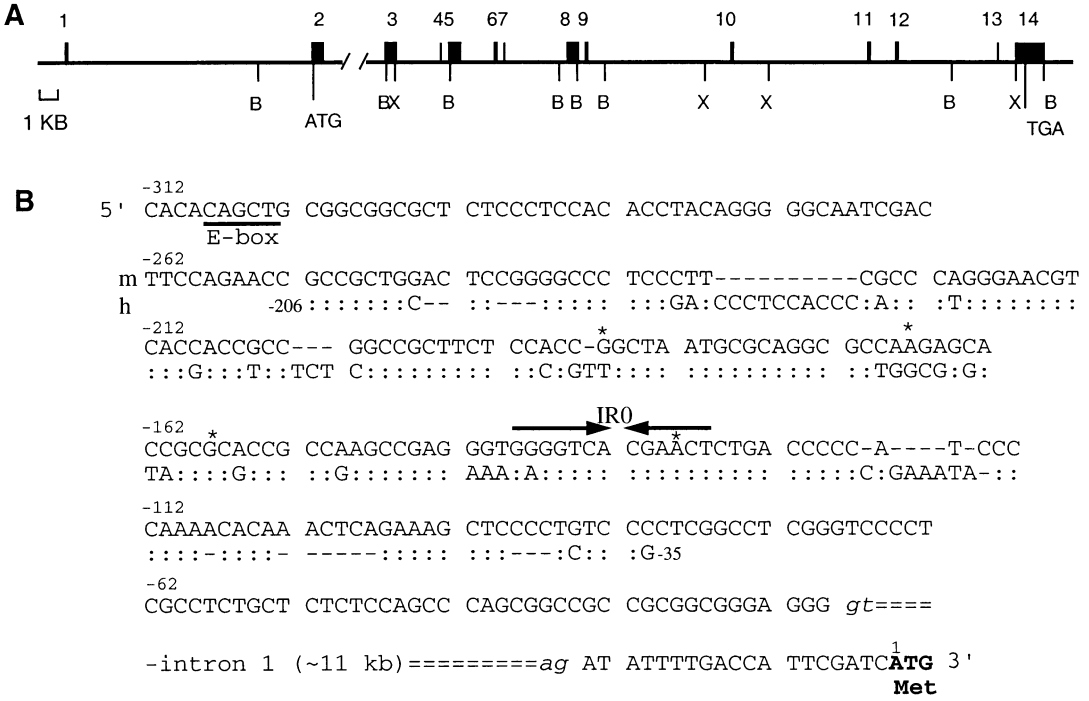

FIG. 1. (A) The entire gene structure of *TR2–11*. The complete 3-kb upstream sequence of this genomic DNA, exon 1, and 250 bp from the splicing donor site of intron 1 were entered into GenBank under the accession number U96095. The numbers above the map indicate the order of the 14 exons. Restriction sites: B, *Bam*HI; X, *Xho*I. (B) Comparison of mouse (top) and human (bottom) *TR2–11* promoters. The translation starting site of the mouse sequence is numbered arbitrarily as position 1 above the sequence. The human sequence is numbered in reference to the transcription initiation site. Four groups of transcription initiation sites identified from 5′-RACE in the mouse gene are labeled with asterisks. The region of intron 1 is indicated, with splicing donor and acceptor sites printed in italic. The putative regulatory element, the E box, identified with the help of the MatInspector program, is underlined and labeled. The recently identified IR0 element is labeled above the sequence. Colons indicate nucleotide identities, and dashes depict gaps introduced to obtain the alignment.

GFP-TR2 fusion vector. Thirty-six hours after transfection, cells were fixed with 4% formaldehyde in PBS, and stained with bis-benzimide (Hoechst 33258, 0.1 μg/mL in PBS) for 30 min at 37°. GFP-positive cells and their nuclear morphology were examined under a fluorescence microscope. The apoptotic cells exhibiting typical nuclear condensation patterns were identified, and the percentage of apoptotic cells was calculated by dividing the number of GFP-positive, apoptotic cells by the total number of GFP-positive cells. Flow cytometry was conducted on a fluorescence-activated cell sorter, FACSCalibur™ (Becton Dickinson). Following transfection, cells were stained with PI (20 μg/mL). GFP-positive single cells were analyzed for DNA content based upon the PI signals. The percentage of subdiploid cell populations was obtained to determine the percentage of apoptotic cells in GFP-positive populations. Four independent experiments were conducted to obtain the means and SD.

Electrophoretic Mobility Shift Assay

The mobility shift assay and nuclear protein isolation were conducted as described previously [13, 16]. The probes were prepared by end-labeling DNA fragments isolated from the basal promoter of the *TR2–11* genomic segment (positions −212 to −42; Fig. 1) with [α-^{32}P]dCTP. Ten micrograms of isolated nuclear protein was incubated with 1 ng of labeled DNA fragments in each 20-μL reaction.

RESULTS

Characterization of Genomic DNA Containing the First Exon of the TR2–11 Gene and Determination of Its Promoter Activity

In our previous studies [8], the genomic segment that contained the entire coding region of mouse *TR2–11* was identified and characterized. The coding region was transcribed from 13 exons spanning a distance of greater than 50 kb. An additional genomic fragment was later found to also hybridize to the probes prepared from an extended 5′ sequence of the cDNA. This observation prompted us to examine the extended 5′ region of the gene. To obtain the complete 5′-untranslated region, 5′- RACE experiments were conducted. By using a TR2–11 specific primer (starting at the 50th nucleotide downstream from the translation initiation codon) and a 5′ anchor primer in a PCR, four groups of hybridizing fragments were obtained, ranging from 237 to 179 bp in length. This result agreed with the primer extension experiment, in which the major transcription initiation site was mapped to a position 208 bp

upstream of the primer used (data not shown). The largest fragment then was used as the probe to screen a mouse genomic DNA library. One genomic clone was obtained, which contained the exon encoding the translation initiation codon determined in our previous study [8], an upstream intron (intron 1) of approximately 11.5 kb, an exon encoding the 5′-untranslated region (exon 1), and an approximately 3-kb sequence of the further 5′-upstream region. The 3-kb, 5′-untranscribed sequence, exon 1, and approximately 250 bp of the intron 1 sequence were determined and entered into GenBank under the accession number U96095. From these results, it was concluded that the *TR2–11* gene contained a total of 14 exons, with most of its 5′-untranslated sequence encoded in an exon approximately 11.5 kb upstream from the second exon, where the translation initiation site was located. The entire *TR2–11* gene structure was updated as shown in Fig. 1A. Figure 1B shows the proximal 5′-untranscribed sequence, exon 1, the splicing junctions for intron 1, and the 5′-untranslated sequence encoded by exon 2, as well as an alignment (with the BLAST 2 Sequences program) [23] of the reported human *TR2–11* promoter sequence [24]. In the proximal 5′-upstream region, no TATA box was found, but multiple transcription initiation sites were identified in the mouse (labeled with asterisks above the sequence). The position of the translation starting site of the mouse gene was arbitrarily numbered as position 1 because of the presence of multiple initiation sites for transcription. We recently identified an RA-responsive inverted repeat sequence with zero spacer (IR0, inverted arrows above the sequence) in the mouse promoter [25]. Within an approximately 300-bp 5′-upstream region, another *cis*-regulatory element, the E box, was identified in a computer search. The E-box sequence has been shown to be a binding site for a basic helix-loop-helix transcription factor [26, 27]. In addition, this region has a very high G/C content (70%, nucleotide numbers -111 to -312). The sequences of the splicing donor and acceptor of intron 1, approximately 250 bp of each, were entered into GenBank under the accession number U28269. By comparing 3-kb sequences of the mouse (this study) and the human [24] gene promoters, the most highly conserved sequence is located in this promoter region, with approximately 78% nucleotide identity.

The presence of a large intron following exon 1 that encoded only the 5′-untranslated sequence prompted us to evaluate the potential regulatory activity of this intron. We first determined whether inclusion of this intron had an effect on the expression of this gene by examining reporters containing this intron or deleted in this sequence. The first reporter contained the *E. coli lacZ* coding sequence inserted in-frame, at the fourth codon in the genomic segment, resulting in the reporter designated as TR2-lacZ/in (containing the entire intron 1). The second reporter was constructed by fusing the *lacZ* coding sequence, in-frame, at the fourth codon of the cDNA, followed by the addition of the same 3-kb 5′-upstream region to the 5′ end of the cDNA, designated as TR2-lacZ/del-in (intron 1 completely deleted). The third reporter was constructed to retain only approximately 250 bp of sequences of the splicing junctions, with most of the intron 1 sequence deleted, and was designated as TR2-lacZ. These constructs are shown in the upper portion of Fig. 2. All three constructs utilized the same transcription and translation control, and varied only at the splicing of intron 1. Therefore, the splicing effect (at intron 1) could be evaluated by comparing the reporter activities of the three constructs. The activities of these reporters were determined in P19 cells, which expressed the endogenous *TR2–11* gene. Each lacZ reporter activity was normalized to an internal control, luciferase activity, as well as to the molecular size of the lacZ reporter to obtain the relative reporter activity of each construct. The relative reporter activity of TR2-lacZ was given an arbitrary value of 100, and the percentile reporter activities of other reporters were calculated by comparing each relative reporter activity with that of TR2-lacZ. The one-sample *t*-test was used to obtain the statistical significance. It appeared that inclusion of intron 1, either as the entire intron (TR2-lacZ/in) or as a truncated intron (TR2-lacZ), enhanced the reporter activity approximately 2- to 3-fold, and the difference between TR2-lacZ/in and TR2-lac was not statistically significant. Therefore, both the 5′-upstream region and the intron 1 splicing sites were required for effective gene activity. In the subsequent experiments, TR2-lacZ (containing the 3-kb 5′-upstream region and intron 1 splicing sites) was used as the parental vector for further promoter deletion constructs as described in the following section.

A series of 5′-deletion reporters were constructed by restriction enzyme digestion of the TR2-lacZ parental vector, resulting in constructs −1938 (*Nco*I deletion), −1738 (*Bgl*II deletion), −1228 (*Hin*dIII deletion), and −388 (*Stu*I deletion). Several smaller reporters designated as −312, −262, −212, and −163 were generated by using PCR-synthesized DNA fragments as shown in Fig. 2. The whole panel of reporters was tested again in P19 cells, and the results from three independent transfection experiments were summarized. It appeared that the −312 reporter encoded the maximal promoter activity, and deletion of 50 bp (the -262 construct), deleting the E-box and its flanking region, reduced its promoter activity by approximately 3-fold ($P < 0.01$). A further 50-bp deletion (the −212 construct) slightly decreased the reporter activity. However, when a further 50-bp sequence was deleted (the −163 construct), the promoter activity was abolished completely. Therefore, the minimal and the maximal promoter activities in this 3-kb regulatory region of the *TR2–11* gene in P19 cells were encoded in a short 212- and 312-bp sequence, respectively, upstream from the translation initiation site.

Induction of TR2–11 Reporter Activities by RA

Our previous data showed that depletion of RA results in drastically decreased TR2–11 expression *in vivo* [10]. Recently, we identified a strongly RA-responding DNA ele-

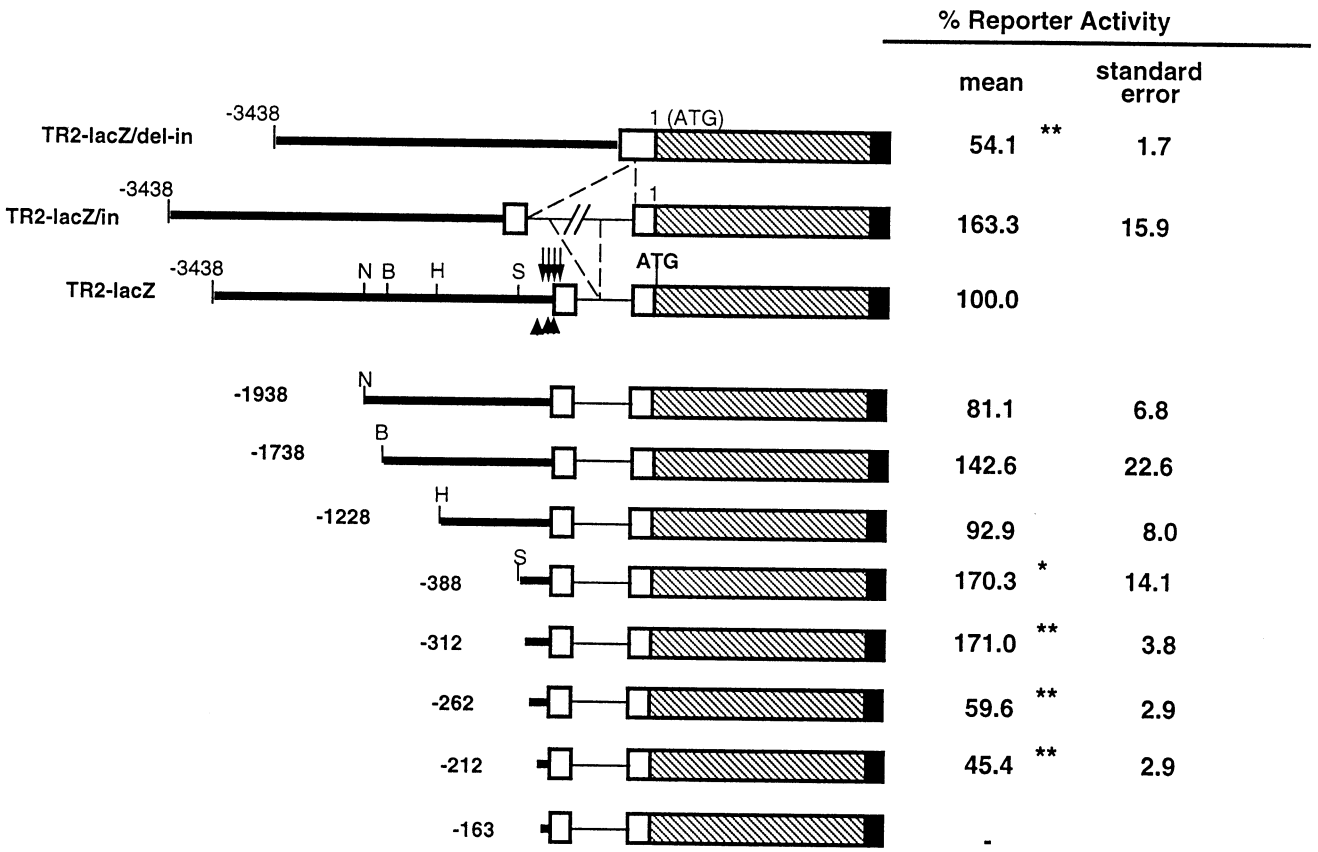

FIG. 2. LacZ reporter constructs and their activities in P19 cells. Hatched bars represent the lacZ sequence, black bars represent the SV40 poly(A) sequence, open bars represent the 5′-untranslated sequence, the thick line represents the 5′-untranscribed sequence, and the thin line represents the intron 1 sequence. Four groups of transcription initiation sites are each labeled with an arrow, and the repeats are indicated with arrowheads. N: *Nco*I; B: *Bgl*II; H: *Hin*dIII; S: *Stu*I. The reporter and internal control vector were added in the amount of 0.4 and 0.05 μg, respectively, per 5×10^4 cells in one well of a 24-well plate. Luciferase activity and lacZ activity were determined. Relative reporter activity was calculated by normalizing the lacZ activity to the luciferase activity, as well as to the molecular size of each reporter. The relative reporter activity of TR2-lacZ was given an arbitrary value of 100, and the percentile reporter activity of each reporter was determined by comparing its relative reporter activity with that of TR2-lacZ. Triplicate cultures were used in each experiment, and three independent experiments were conducted to obtain the means and SEM. The significance of differences was determined using the one-sample *t*-test (StatView 4.5). Key: (*) $P < 0.05$; and (**) $P < 0.01$.

ment, IR0, from this promoter region [25]. Then, we asked whether RAR and RXR play a role in the transcriptional control of this gene by using reporters driven by its natural promoter with various sizes of 5′ regulatory region. Since the endogenous RARs/RXRs were present abundantly in P19 cells, it was difficult to assess the effect of additional RAR/RXR on the reporter activities in P19 cells. Therefore, COS-1 cells, with a much lower background of endogenous nuclear receptors, were used in the following transfection experiments. COS-1 cells were co-transfected with TR2-lacZ deletion constructs (as shown in Fig. 2), the RAR and RXR expression vectors, and a luciferase vector for transfection control. Then RA was added to the culture medium, and the relative reporter activity was determined. As shown in Fig. 3A, all the constructs that encoded an active reporter activity were induced by RA up to 4-fold. In experiments where RAR/RXR expression vectors were replaced with the control expression vectors, no induction was detected (data not shown), indicating that RA induction of these reporters was mediated by exogenously provided RAR/RXR. In addition, RA induction was effective on the minimal promoter of the *TR2–11* gene.

In our recent studies [25], we provided evidence that RAR/RXR dimers are able to bind to the dissected IR0 oligonucleotide fragments with a high affinity in gel shift experiments, and mutations in either one of the two repeats completely abolish RA responsiveness of reporter genes and RAR/RXR binding to these fragments. To provide further evidence for direct RAR/RXR binding to this DNA fragment in its natural genomic context, a gel mobility shift assay was conducted by using the minimal promoter DNA fragment (−42 to −212) as a probe. Nuclear extracts were isolated from COS-1 cells, which contain negligible endogenous RAR/RXR, as well as COS-1 cells transfected with RAR/RXR expression vectors. The extracts were incubated with ^{32}P-labeled DNA fragments and resolved on a polyacrylamide gel. As shown in Fig. 3B, little DNA binding activity was detected in nuclear extracts isolated from the

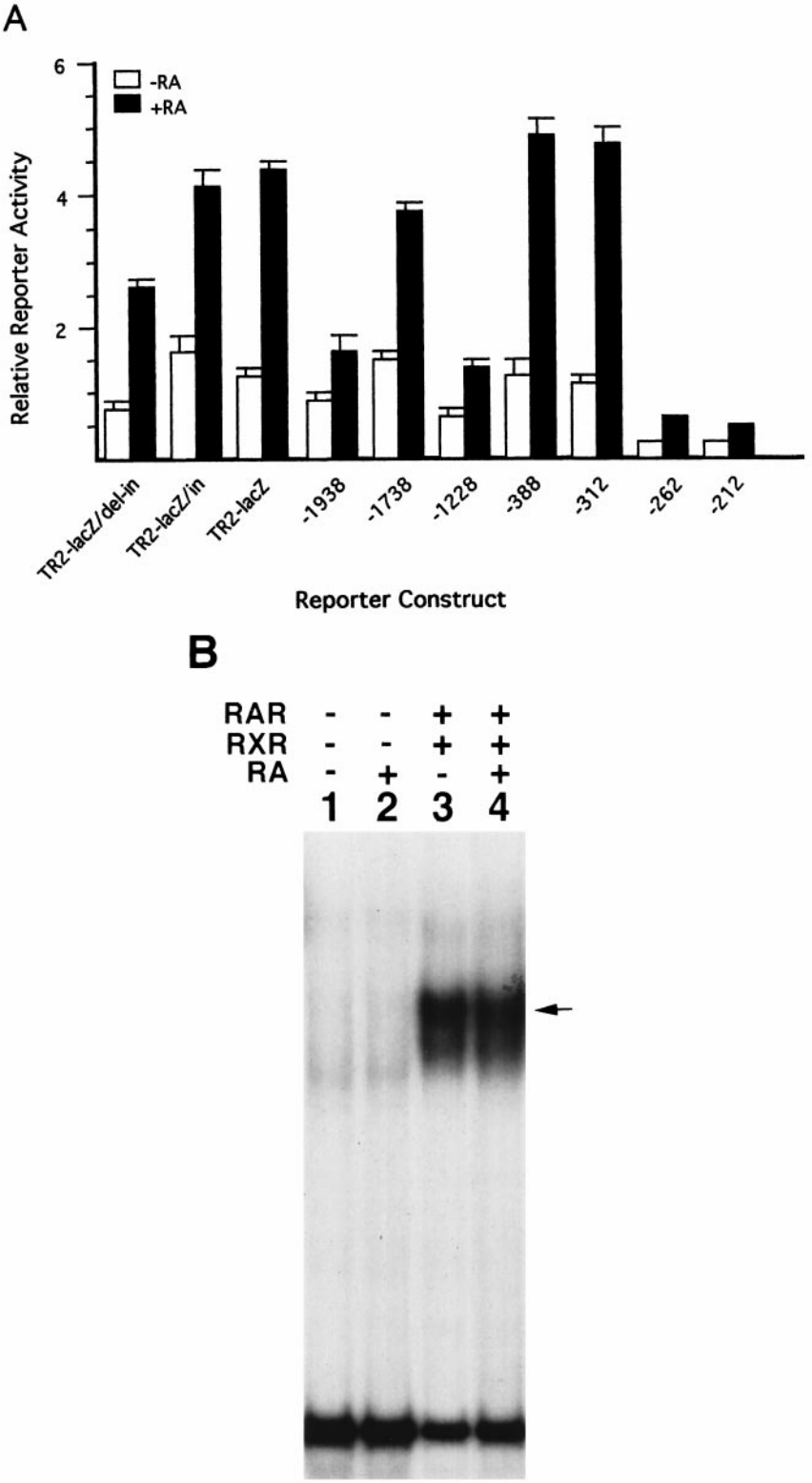

FIG. 3. RAR/RXR-mediated RA induction of reporter activities in COS-1 cells. (A) COS-1 cells were plated in dextran–charcoal-treated (DCC) medium and co-transfected with a reporter (0.4 μg TR2-lacZ or its deletion mutants), an internal control luciferase vector (pGL3, 0.05 μg), and nuclear receptor expression vectors RAR (0.1 μg) and RXR (0.1 μg) in each well. at-RA (5×10^{-7} M) was added to the cultures 12 hr later, and the lacZ (specific reporter) and luciferase (for transfection control) activities were determined 24 hr following the addition of RA. Relative reporter activity was calculated by normalizing the lacZ activity to the luciferase activity. The open and solid bars represent experiments without and with RA treatment, respectively. The standard errors are not shown in some of the experiments because of the scale of the y-axis. (B) Gel mobility shift experiment using nuclear proteins from COS-1 cells. Nuclear proteins isolated from COS-1 cells transfected with either control or RAR and RXR expression vectors were examined for DNA binding. The upper portion of the panel shows the expression vectors or RA added to the cells.

control COS-1 cells with or without the addition of RA (lanes 1 and 2), whereas intense bands were detected in reactions using nuclear extracts isolated from COS-1 cells transfected with RAR/RXR expression vectors. This result was confirmed in experiments using *in vitro* translated RAR/RXR protein preparations (data not shown). These data confirmed that the *TR2–11* minimal promoter was a direct target for RAR/RXR binding.

RA Regulation of TR2–11 in P19 Cells

Data described above showed that the expression of reporters driven by the *TR2–11* promoter was induced by RA, and this promoter could be bound by RAR/RXR. The physiological significance of this observation then was addressed in an RA-responding cell line that expressed endogenous RAR/RXR and TR2–11. P19 cells were maintained in exponentially growing cultures, and RA (5×10^{-7} M concentrations of either at- or 9c-RA) was added. RNA samples were collected at different time points following the addition of RA and analyzed on a Northern blot by using a TR2–11-specific probe (the ligand-binding domain), as well as an actin-specific probe. For a positive control of RA induction, expression of an early RA responsive gene, RAR_β, was examined on a duplicate blot. As expected, the RAR_β gene was induced readily by RA within 6 hr following RA treatment (Fig. 4A, lower panel). The induction of TR2–11 appeared later, approximately 24 hr after the addition of at- or 9c-RA (Fig. 4A, upper panel). Prolonged RA treatment had no further inducing effects on the expression of this gene (data not shown). Therefore, the endogenous *TR2–11* gene could be induced by RA in P19 cells.

To verify whether the relatively weak and late RA responsiveness of the *TR2–11* gene, as compared with the early induction of RAR_β, was a natural property of this promoter in the context of P19 cells, we examined the activities of TR2–11 and RAR_β reporters in P19 cells without adding exogenous RAR/RXR. As the RA-response region of the *TR2–11* gene was located in its minimal promoter region (Fig. 3A), the -212 reporter (Fig. 2) was examined and compared with the reporter controlled by the minimal, natural RAR_β promoter that contained a DR5 RARE [21]. An internal control, the tk-luciferase reporter, was included in each transfection. At different time points following the addition of RA, cells were harvested, and the reporter activity was determined. In agreement with the results of Northern blot analysis (Fig. 4A), induction of the TR2–11 reporter (−212 construct) was detected 24 hr after RA treatment (Fig. 4B, columns 1–6), whereas the RAR_β reporter was induced readily within 12 hr (Fig. 4B, columns 7–12). These data suggested that the relatively weak and delayed induction of TR2–11 probably resulted from complex interactions of nuclear factors that regulated TR2–11 expression in the P19 background.

Therefore, it was speculated that RA induction of this gene might also be regulated by other nuclear factors, in addition to RAR/RXR. To examine this possibility, RA induction experiments were repeated in the presence and absence of a protein synthesis inhibitor, cycloheximide (Fig. 4C). In agreement with the Northern blot studies, TR2–11 mRNA was induced weakly by RA at 12 hr (lane 2) and induced maximally by RA at 24 hr (lane 3). Interestingly, cycloheximide alone elevated the level of TR2–11 expression (lanes 4 and 5), indicating that some negative factors for the expression of this gene were inhibited by blocking protein synthesis. Furthermore, RA induction of TR2–11 occurred in the presence of cycloheximide (lanes 6 and 7), and the induction, in fact, was effective at an earlier time point (12 hr, lane 6). This result indicated that the inducing effect of RA required no protein synthesis and was effective immediately when other negative nuclear factors (or destabilizing factors) were blocked by the protein synthesis inhibitor. Therefore, RA regulation of TR2–11 in P19 cells was a direct event mediated by endogenous RAR/RXR, but other nuclear factors, mostly negative, also played a role in the control of expression of this gene.

Apoptosis of P19 Cells Overexpressing TR2–11

RA was capable of inducing P19 differentiation accompanied by apoptosis [28–31]. The RA-induced DNA fragmentation, a hallmark of apoptosis, became obvious 24–36 hr after drug treatment [31], which corresponded to the time when TR2–11 was accumulated maximally. To investigate whether TR2 elevation had an effect on cellular apoptosis of P19, P19 cells were transfected with a GFP-tagged TR2 protein (the GFP-TR2 fusion). The GFP-TR2 fusion had been engineered previously by placing the GFP coding sequence at the N-terminus of the TR2–11 coding region. This fusion retained the characteristics of TR2–11, such as nuclear localization, *trans*-repressive activity, and dimerization on the target DNA element [13]. The GFP control vector also was introduced into P19 as a transfection control. Thirty-six hours after transfection, cells were fixed and stained with bis-benzimide (Hoechst 33258), a DNA-specific fluorescent dye [32, 33]. The GFP-positive cells and apoptotic cells were identified under a microscope. The apoptotic cells were stained with the DNA dye in a typical nuclear condensation pattern. It appeared that many of the GFP-positive cells in GFP-TR2-transfected cultures were apoptotic, as shown in panels A and B of Fig. 5. In the GFP-transfected control cultures, most GFP-positive cells displayed a healthy nuclear morphology as shown in panels E and F of Fig. 5, indicating that the expression of GFP had no significant effects on apoptosis. By counting GFP-positive cells that were also apoptotic in the two transfected cultures, it was estimated that an average of 50% of GFP-TR2-positive cells were undergoing apoptosis, whereas only approximately 15% of control cells (GFP-positive) were apoptotic as a result of the transfection procedure (open bars in Fig. 5G). To quantify apoptotic cells better, the transfected cultures were analyzed in a

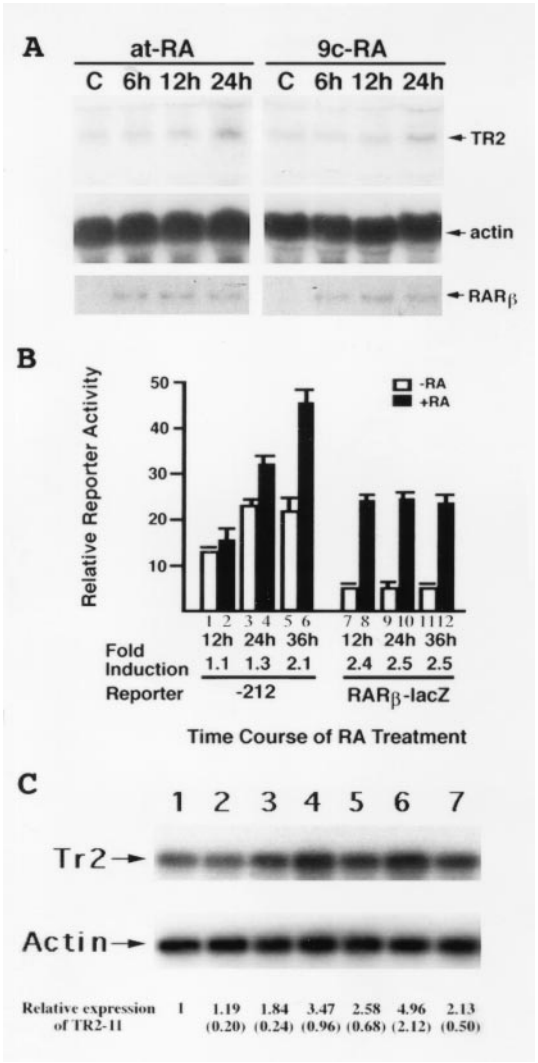

FIG. 4. Induction of TR2–11 expression by RA in P19 cells. (A) Northern blot analyses of endogenous TR2–11 expression induced by RA. P19 cells were treated with RA (at- or 9c-RA, 5×10^{-7} M) or vehicle. RNA was isolated at different time points for northern blot analysis (15 μg/lane) of TR2–11 and actin expression. Detection of RAR_{β} gene expression (lower panel) on a duplicate blot was included as a positive control for RA induction. RNA from the control culture was harvested 24 hr after vehicle treatment. (B) RA induction on reporters driven by the natural promoters. P19 cells were transfected with lacZ reporters (0.5 μg) driven by the minimal, natural promoter of *TR2–11* (-212 construct) or RAR_{β} (RAR_{β}-lacZ), together with a tk-luciferase internal control (0.1 μg). Twelve hours after transfection, RA was added at a final concentration of 5×10^{-7} M, and cells were harvested at different time points. Fold RA induction is indicated below the graph. (C) RT–PCR analyses of TR2–11 expression in RA and cycloheximide-treated cultures. P19 cells were treated with RA alone for 12 hr (lane 2) and 24 hr (lane 3), cycloheximide alone (2 μg/mL) for 12 hr (lane 4) and 24 hr (lane 5), and a combination of RA and cycloheximide for 12 hr (lane 6) and 24 hr (lane 7). RNA was analyzed by RT–PCR and quantified as described in the text. The normalized TR2–11 expression level of the control culture (lane 1) was arbitrarily set at a value of 1 to determine the relative expression level of TR2–11 in the drug-treated cultures. The means and SD (in parentheses) were obtained from three independent experiments and shown under the representative blot.

FACS apparatus. Cellular DNA was labeled with PI, and cells with a subdiploid DNA content were identified. The fraction of subdiploid cells among all the GFP-positive cells was determined to obtain the percentage of apoptotic cells that captured the exogenously provided TR2–11. The results are indicated by the black bars in Fig. 5G, which were in good agreement with the results of bis-benzimide staining (empty bars). Therefore, it appeared that overexpression of TR2–11 in P19 rendered approximately 50% of the cells apoptotic. Interestingly, the increase in P19 apoptosis (as a result of transfection with GFP-TR2) was not observed in COS-1 cells (data not shown), indicating a cell type-specific effect of TR2 overexpression on apoptosis. These data suggested that TR2–11 might be one of the downstream effectors for RA-induced P19 apoptosis.

DISCUSSION

This study describes the regulatory region of the mouse orphan receptor *TR2–11* gene, its regulation by RA, and the potential role of TR2–11 in RA-induced P19 apoptosis. This gene contained a total of 14 exons, with the first exon encoding most of its 5′-untranslated sequence. The first exon was located approximately 11.5 kb upstream from the second exon, where a translation initiation site was present. In the GC-rich, 5′ proximal region, multiple transcription initiation sites were found. The regions encoding the maximal and the minimal promoter activities in P19 cells were located within positions -312 and -212, respectively. The activities of reporters driven by the natural *TR2–11* promoter could be induced by RA in COS-1 cells supplied with exogenous RAR and RXR. Furthermore, *in vitro* translated RAR/RXR or nuclear extracts isolated from COS-1 cells transfected with RAR/RXR expression vectors could bind to this minimal promoter fragment. Consistent with this result, this gene was induced by RA in the P19 cell line, which expresses endogenous RARs/RXRs, and overexpression of this protein induced P19 apoptosis in the absence of RA, suggesting a functional role of TR2–11 in RA-induced apoptosis of P19.

By dissecting the 5′-upstream regulatory region of the *TR2–11* gene, the RA-response sequence was located within its minimal promoter region, where an imperfect palindromic IR0 (position −138 to −127, GGGTCA CGAACT) has been demonstrated to be a functional RA response element [25]. The human *TR2* gene structure has been examined [24] and shown to be similar to the mouse *TR2* gene structure [8]; however, the biological activity of the human *TR2* gene promoter has not been demonstrated [24]. This study provides the first evidence for the biological activity of the mouse *TR2* gene promoter as well as its regulation by RA. By extending from our previous studies [25], which dissected the RA-responding IR0 element from the *TR2–11* promoter, this study has demonstrated a physiological relevance of RA regulation on the expression of the *TR2–11* gene in its natural genomic context.

The fact that TR2–11 suppresses the expression of many

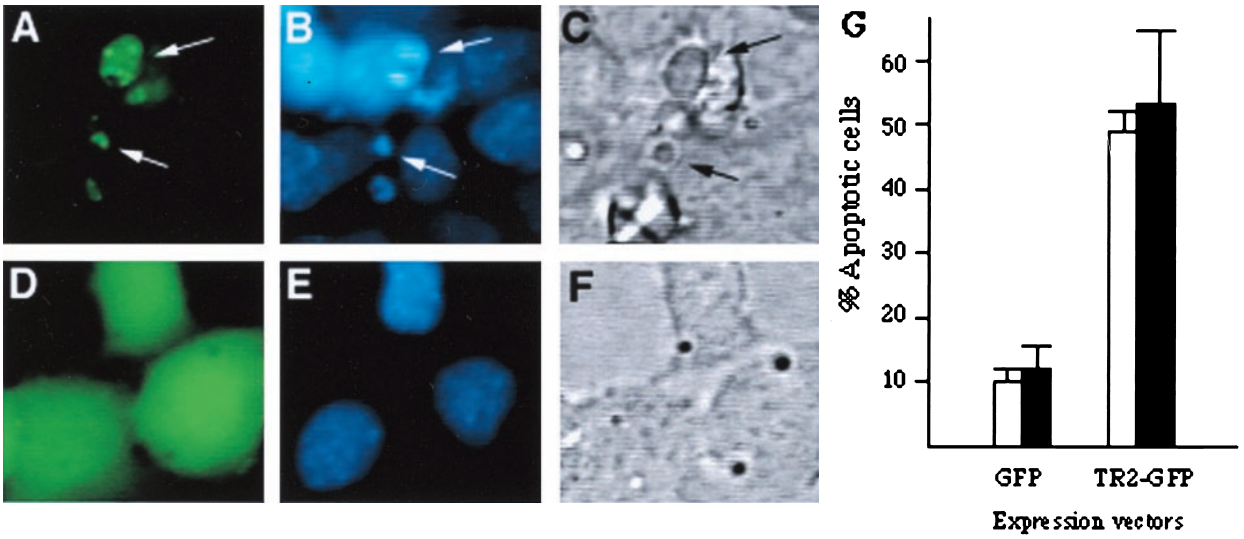

FIG. 5. Apoptosis in P19 cultures overexpressing the GFP-TR2 fusion protein. P19 cells were transfected with either the GFP control (D–F) or the GFP-TR2 fusion expression vector (A–C). Thirty-six hours after transfection, cells were fixed and stained with bis-benzimide and observed under a microscope. Panels A and D show the GFP images under a fluorescence filter, panels B and E show the bis-benzimide stained nuclear morphology under a UV filter, and panels C and F are images under a bright-field filter. GFP–TR2 was located in the nuclei, while GFP alone was distributed evenly inside the cells. Apoptotic cells with obvious apoptotic bodies are indicated with arrows. (G) Statistical analyses of apoptotic cells. The open bars show the statistical results of bis-benzimide-stained cells examined under a fluorescence microscope. The solid bars show the statistical results of PI-stained cells from FACS analyses. Values are means ± SD obtained from four independent experiments.

RA-target genes [12–18] would suggest that the weak and delayed induction of TR2–11 by RA could be physiologically significant. An analogy can be found in the insect steroid hormone ecdysone system, which is essential for the embryogenesis of *Drosophila* [34]. The effects of ecdysone are mediated by its nuclear receptor, the heterodimer of Ecr and Usp. An autoregulatory loop, similar to the RA induction of several retinoid receptor genes in mammals, is also found in this insect hormone system. Among several downstream effectors of the Ecr/Usp signaling pathway, the nuclear receptor DHR78, which shows high homology to TR2–11, is categorized as a late response gene and can antagonize the activity of Ecr/Usp. In the case of TR2–11, several studies of ours [12–14, 16] as well as others [15, 17, 18] all have shown a negative regulatory activity of TR2–11 on RA signaling pathways either by competing for DNA binding or by differential interaction with a common coregulator, RIP140. In a scenario proposing that TR2–11 plays an antagonistic role in RA signaling, its expression would have to be regulated. Many genes induced by RA are important players in cellular processes. If TR2–11 is induced strongly by RA, it could immediately shut down the expression of these genes that are required for important cellular events. On the other hand, if RA induction is left unchecked, cells would undergo catastrophic changes. The experiments shown in Fig. 4C strongly support the hypothesis that the expression of TR2–11 is also regulated by other negative factors that are blocked by cycloheximide. Therefore, it is tempting to speculate that TR2–11 may act to fine-tune RA induction and serve as a checkpoint for RA-induced cellular processes.

Both RAR- and RXR-specific agonists have been shown to induce cellular apoptosis accompanied by differentiation in P19 cultures, and programmed cell death becomes evident approximately 24 hr after the administration of drugs, as determined by nuclear condensation or DNA fragmentation [35]. TR2–11 is also up-regulated by RA at the highest level within 24 hr, in agreement with the time of apparent apoptosis of RA-induced P19 cultures. Most interestingly, ectopic expression of TR2 in transiently transfected P19 cells results in a dramatic increase of apoptotic cells. Therefore, it is also tempting to suggest a functional role of TR2–11 in mediating RA-induced apoptosis of P19 cultures. The mechanism of this apoptotic process induced by overexpression of TR2–11 remains to be elucidated. It will be interesting to examine the role of TR2–11 in the control of several apoptotic factors such as the bone morphogenic proteins [36].

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