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Identification of a Retinoic Acid Response Domain Involved in the Activation of the β_1-Adrenergic Receptor Gene by Retinoic Acid in F9 Teratocarcinoma Cells

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ABSTRACT. The density of β_1-adrenergic receptors (β_1-AR) is up-regulated upon differentiation of embryonic F9 teratocarcinoma cells by retinoic acid (RA) to the primitive endodermal phenotype. To identify the domains involved in RA-mediated activation of β_1-AR gene transcription, three kb of 5′-flanking sequence of the β_1-AR gene were ligated to a luciferase reporter gene and transiently transfected into F9 cells that were pre-exposed to 100 nM RA for 2 days. By generating deletions in the β_1-AR promoter, a region between −125 and −100 was found to mediate a 3-fold induction in cells exposed to RA for an additional 2 days. Through site-directed mutagenesis of this region, it was determined that the RA responsive element (RARE) was organized as a direct repeat separated by 5 nucleotides in which the 5′-most AGGTCG half-site was between nucleotides −106 and −101 and the 3′-most AGGTCA half-site was between nucleotides −117 and −112. The RA receptor α (RARα) isoform bound to the oligomer representing the sequences between −125 and −100 as a heterodimer complex with the retinoid X receptor α (RXRα). In a separate study, it was determined that the nucleotides between −125 and −100 are involved in thyroid hormone-mediated activation of the β_1-AR gene in ventricular myocytes. Therefore, transcriptional activation of the β_1-AR gene by thyroid hormone or RA involves a single binding site in the promoter. BIOCHEM PHARMACOL **55**;2:215–225, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. β_1-adrenergic receptors; retinoic acid; teratocarcinoma cells; promoter analysis; retinoic acid responsive domain; gene expression

Vitamin A (retinol) and its biologically active derivatives (collectively known as retinoids) play an important role in development, metabolism, cellular differentiation, and proliferation [1]. Except for vision, the biological actions of vitamin A (retinol) are believed to require its metabolic conversion to RA†, a process that appears to be regulated by cellular retinol binding proteins [2]. Retinoids exert their numerous effects by binding to RAR. RARs belong to the superfamily of nuclear ligand activated transcription factors that include steroid hormone, thyroid hormone, and vitamin D_3 receptors [3]. Retinoid signaling is diversified due to the existence of two families of RA receptors, the RARs and the RXRs [4, 5]. Each of these families consists of three isotypes (α, β, and γ) that are encoded by separate genes. Further complexity in the RAR/RXR family is generated by alternative promoter usage and differential splicing [5]. For example, the two isoforms of RARα (RARα1 and α2) have different amino termini that arise from the use of different first exons [5]. Both all-*trans* RA and 9-*cis* RA are ligands for the RAR family, whereas the RXR family is activated exclusively by 9-*cis* RA [5].

RARs and RXRs form heterodimers that bind to specific DNA sequences known as RAREs and regulate transcription in a ligand-dependent manner. RXRs enhance the binding not only of RARs, but also of thyroid hormone and vitamin D_3 receptors to their responsive elements [6]. The RAREs that have been identified in the regulatory regions of target genes whose transcription is induced by RA consist of a direct repeat of 5′ (A/G)G(G/T)TCA) separated by a 5-bp (DR5) or 2-bp (DR2) spacer [4–6]. Such genes are optimally activated by RXR-RAR heterodimers, with binding of the ligand to the RAR moiety sufficient for activation.

In F9 mouse teratocarcinoma stem cells, RA induces the differentiation of these cells to a cell type that functionally resembles primitive endoderm [7]. In RA-differentiated cells, the secretion of tissue plasminogen activator and the density of β-adrenergic receptors are increased markedly [8, 9]. The density of β-adrenergic receptors in primitive

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† *Abbreviations:* RA, retinoic acid; β_1-AR, β_1-adrenergic receptor; RAR, retinoic acid receptor; RARE, retinoic acid response element; RXR, retinoid X receptor; DR5, direct repeat separated by 5 oligonucleotides; TSS, transcriptional start site; and PEPCK, phosphoenolpyruvate carboxykinase.

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endodermal (RA-treated) F9 cells is 3-fold higher than that in unstimulated F9 cells [9]. The β_1-AR comprises 71% of the total β-adrenergic receptors in F9 stem cells, and the proportion of β_1-AR increases to 86% in RA-treated primitive endodermal cells. These observations indicate that RA significantly induces the β_1-AR subpopulation in these cells.

The β_1-AR is encoded by an intronless and TATA-less gene that is rich in GC sequences in the first 0.5 kb of the 5′-flanking region [10–15]. The TSS in the rat β_1-AR genes occurs 253 bp 5′ to the initiator ATG, whereas in the ovine and murine β_1-AR genes the TSS occurs 415 and 660 bp 5′ to the ATG, respectively [12, 14, 15]. Several domains in regions that are 5′ and 3′ to the TSS were identified as regulators of basal expression of the β_1-AR gene. A putative Sp1 site between −386 and −366, which enhances the transcription of the β_1-AR gene, has been described [12], while the sequences between −125 and −100 suppress basal transcription [16]. A glucocorticoid responsive domain between −950 and −926 relative to the initiator ATG suppresses the expression of the β_1-AR gene in response to glucocorticoids [17].

The goal of our studies was to characterize the *cis*-acting sequences in the rat β_1-AR promoter and *trans*-acting proteins involved in RA stimulation of β_1-AR transcription. Our results indicate that RA stimulates β_1-AR transcription through a direct repeat AGGTCA-like motif between nucleotides −100 and −125 and that the RAR can bind this sequence as a heterodimer with RXR.

MATERIALS AND METHODS

Preparation of Recombinant Proteins and F9 Nuclear Extracts

Human RARα cDNA in the pET8c plasmid was transformed into *Escherichia coli* BL21(DE3)plysS cells [18]. The cells were induced with 0.4 mM isopropyl-D-β-thiogalactopyranoside for 3 hr, and the crude bacterial lysate was used in gel-shift experiments. The full-length cDNA of the human RARα was ligated into the *Kpn*I and *Sma*I sites of pQE32, which introduced a histidine tag on the amino terminus (Qiagen). The cDNA for RXRα [19] was digested with the *Nco*I site at amino acid 27 and *Hin*dIII at a site in the untranslated region of RXRα, and the resulting insert was ligated into the vector pQE9. Histidine-tagged RARα and RXRα were expressed in the BL21(DE3) *E. coli* strain. Following induction for 1.5 hr with 1.0 mM isopropyl-D-β-thiogalactopyranoside, the *E. coli* were harvested and lysed by repeated freeze thawing. The particulate material was removed by centrifugation at 100,000 × *g* for 60 min in a Ti80 rotor. Further purification was performed by binding the histidine-tagged receptors to a nickel affinity resin (Qiagen) containing 10 mM Tris (pH 7.5), 400 mM KCl, 1 mM β-mercaptoethanol, 20% glycerol, and protease inhibitors. After washing the resin with buffer containing either 1, 10, or 40 mM imidazole, the histidine-tagged receptors were eluted with 200 mM imidazole [20]. The purity of the protein prepared by this method exceeded 90%, as determined by silver staining.

F9 extracts from F9 cells grown in the absence or presence of 100 nM RA for 5 days were prepared by the method of Dignam *et al.* [21].

Gel Mobility Assays

Double-stranded oligomer corresponding to the sequence between −125 and −100 in the β_1-AR promoter and flanked with *Sac*II-compatible ends, CTAGAGGCTGC CCTGACCTGGCCGCGACCTCT, was labeled with Klenow enzyme and [α-^{32}P]dCTP [20]. The binding reactions were performed at room temperature for 20 min in buffer containing 80 mM KCl, 10 mM HEPES (pH 7.1), and 10% glycerol. Each binding reaction contained between 1 and 2.5 μg of poly(dI · dC) as nonspecific competitor, proteins, and antisera as indicated. The sequence of the competing optimal RARE was 5′-CTA GAAGGTCACGCAGAGGTCAT-3′ [22]. The resulting complexes were resolved on 5% nondenaturing acrylamide gels in 25 mM Tris, 200 mM glycine at 4° [23]. The antiserum to RARα (C-20) was purchased from Santa Cruz Biotechnology.

Construction of Luciferase Vectors

The β_1-AR *Kpn*I-*Sac*II segment extending from −1250 to −126, relative to the initiator ATG, was subcloned into pBluescript SK$^+$ and then excised with *Kpn*I and *Sac*I and cloned into pGL3*basic* (Promega). pGL3*basic* is a promoterless luciferase expression plasmid containing a multiple cloning site 5′ to the luciferase insert. To generate the −1250 to −100 pGL3*basic* plasmid, a 28-bp double-stranded oligomer GGCTGCCCTGACCTGGCCGC GACCTCGC encoding the region from −125 to −100 in the β_1-AR promoter and flanked with *Sac*II-compatible ends was ligated into the *Sac*II site. This insertion created the sequence of the β_1-AR promoter from −1250 to −100 driving the luciferase reporter gene. The −3311 to −126 and −3311 to −100 plasmids were generated by ligating the *Eco*RI-*Kpn*I fragment extending from −3311 to −1251 5′ to the *Kpn*I site in the appropriate pGL3*basic* plasmid.

To introduce mutations into the −125 to −100 region, six pairs of 28-bp oligonucleotides containing the appropriate mutations and flanked with *Sac*II-compatible ends were synthesized. The annealed oligonucleotides were subcloned into the *Sac*II site at −126 in the [−3311, −126]pGL3*basic* vector. Plasmids containing a single copy of the insert in the proper orientation were identified by sequencing.

To test whether the sequences between −125 and −100 in the β_1-AR promoter could confer hormonal responsiveness on an enhancerless gene, the 32-bp oligomer flanked by *Xba*I-compatible ends was cloned into the *Nhe*I site in the multiple cloning region of the pGL2*promoter* vector (Promega). The pGL2*promoter* vector is an enhancerless luciferase vector driven by the neutral SV40 promoter. Two

plasmids were generated, one containing a single copy of the oligomer in the proper orientation and the other containing a concatemer of three copies, all of which were in the reverse orientation.

To mutate the sequences between −126 and −100 in isolation of the full-length promoter, seven pairs of 32-bp oligonucleotides GATCTGGCTGCCCTGACCTGGCCGC GACCTCA containing the appropriate mutations and flanked with *Bgl*II compatible ends were synthesized. The underlined sequence is that found between nucleotides −125 and −99 in the β_1-AR gene. The annealed oligonucleotides were subcloned into the *Bgl*II site in the multiple cloning region of the pGL2*promoter* vector. Plasmids containing a single copy of the insert in the proper orientation were identified by sequencing. The nomenclature of the β_1-AR constructs is [5′ end, 3′ end] to indicate the 5′ and 3′ boundaries of a DNA segment. The numbers in brackets (see Table 2) reveal the localization of each segment relative to the translational start site of the rat β_1-AR gene [10, 11].

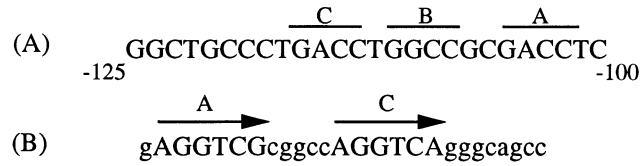
Cell Transfections and Luciferase Assays

F9 cells were cultured on gelatin-coated dishes in Dulbecco's Modified Eagle's Medium containing 15% fetal bovine serum that was pretreated twice with activated charcoal and dextran [24]. Plasmids were introduced into these cells by calcium phosphate precipitation [24, 25]. F9 cells were seeded at a density of 1×10^5 cells/60-mm dish. After 24–48 hr, each dish was exposed to 100 nM RA or buffer (2 mM HEPES, pH 12) for 48 hr. Each dish was transfected with 13 μg of plasmid DNA composed of 5 μg of the smallest vector (pGL3*basic*), 6 μg of carrier pGEM7ZF$^+$ DNA, and 2 μg of simian virus (SV40)-β-galactosidase (pSV-β*gal*, Promega) as a transfection control. In all transfections, the amount of each β_1-AR-luciferase construct was increased to an equivalent molar ratio of pGL3*basic*, and the balance of the DNA was adjusted to 6 μg with pGEM7ZF$^+$. Cells were exposed to the calcium phosphate precipitates for 16–20 hr, washed twice with phosphate-buffered saline, and recultured without or with 100 nM RA for an additional 48 hr. Cells were harvested in 250 μL lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, and 1% Triton X-100) and the lysates clarified by centrifugation. In all experiments, the appropriate pGL*control* vector, which was a luciferase vector driven by the SV40 promoter and enhancer sequences, was transfected in equimolar amounts to the promoterless pGL*basic* vector. For each batch of cell lysates, a standard curve was generated by measuring the luminescence generated by 5–5000 fg of firefly luciferase, and the luminometric reading of each sample was converted to picograms of firefly luciferase per milligram of protein [26]. These data provided a measure for the absolute luciferase activity of each construct in each cell type in order to compare the relative activities of the constructs among the different cell lines. Each construct was transfected into three dishes, and these transfections were replicated for each cell type in a minimum of three separate experiments ($N \geq 9$). The values from all experiments were combined, and significance was determined by Student's *t*-test ($P = 0.05$). Luciferase assays were performed using 20 μL of lysate and 100 μL of luciferase assay reagent (Promega) injected automatically into a Turner-20 luminometer. β-Galactosidase assays were performed using 150 μL of lysate and the *o*-nitrophenyl-β-D-galactopyranoside substrate as described [27]. Protein was measured in 7.5 μL of extract using a detergent-compatible protein assay (Bio-Rad).

RESULTS

Identification of the RA-Responsive Domain in the β_1-AR Promoter

Transcription of the β_1-AR gene is induced rapidly by RA in F9 cells [9]. Therefore, our initial experiments were designed to identify the region in the β_1-AR promoter required for inducing β_1-AR transcription by RA. Sequence analysis identified a domain between nucleotides −125 and −100 relative to the start of translation of the β_1-AR as a potential RARE because it contained multiple imperfect AGGTCA hexamer half-sites (Fig. 1, A and B). To determine if this region conferred RA responsiveness on the β_1-AR gene, transient transfection experiments were conducted in F9 cells with chimeric genes containing the β_1-AR promoter driving the luciferase reporter gene. Sequences extending from −3311 to either −126 or −100 of the β_1-AR promoter were ligated in front of the luciferase reporter gene in the promoterless luciferase vector pGL3*basic* (Table 1). These vectors were transiently transfected into F9 cells. Because the effect of RA on β_1-AR expression occurred in the primitive endodermal F9 phenotype, F9 cells were exposed to 100 nM RA or buffer for 2 days before DNA transfection. Then the cells were transiently transfected and re-exposed to either 100 nM RA or buffer for an additional 48 hr. Transcription from the promoter extending from −3311 to −126 was not increased by RA in both phenotypes of F9 cells, indicating that there are no functional RAREs in this segment (Table 1). Transcription from the sequences between −3311 and −100 was increased 3-fold in F9 cells that were exposed to RA before and after DNA transfection. When F9 stem cells are treated with RA, the cell growth declines as the number of cells that have terminally differentiated increases [28]. The doubling time of F9 stem cells increased from a mean of 20 ± 5 to 30 ± 6 hr after exposure to 100 nM RA ($N = 3$). Therefore, all luciferase activities were corrected for the protein concentration in the lysate to adjust for differences in cell number within the different samples. The induction of transcription by RA of the β_1-AR-luciferase vector containing nucleotides −3311 and −100 in F9 cells that were exposed to RA after DNA transfection was feeble and not statistically significant (Table 1). However, when luciferase activity was determined 5 days after continuous exposure to RA, the stimulation of transcription by RA of



(C)

Mutations within the -125, -100 region in the β_1-adrenergic receptor promoter	β_1-adrenergic receptor luciferase vector	F9 cells —RA	F9 cells +RA
	Luciferase activity as percent of pGL3control		
GGCTGCCCTGACCTGGCCGCGACCTC	-3311, -100 wt-*Luc*	5 ± 1	17 ± 3*
GGCTGCCCTGACCTGGCCGCATTATC	-3311, -100 (Mut -102/-105) [A]	2 ± 1	2 ± 1
GGCTGCCCTGACCTTAATGCGACCTC	-3311, -100 (Mut -109/-111) [B]	2 ± 1	6 ± 2*
GGCTGCCCTATTATGGCCGCGACCTC	-3311, -100 (Mut -113/-116) [C]	0.1 ± 0.1	0.1 ± 0.1
GGCTGCCCTGACCTTAATGCATTATC	-3311, -100 (Mut -102/-111) [A, B]	1 ± 1	2 ± 1
GGCTGCCCTATTATGGCCGCATTATC	-3311, -100 (Mut [-102/-105] —[-113/-116]) [A, C]	1 ± 1	2 ± 1
GGCTGCCCTATTATTAATGCATTATC	-3311, -100 (Mut -102/-116) [A, B, C]	1 ± 1	1 ± 1

FIG. 1. Effect of mutating the sequences between −125 and −100 in the β_1-AR promoter on its responsiveness to RA. (A) Sequence of the nucleotides between −125 and −100 in the β_1-AR gene. (B) Complementary sequence of the −125 to −100 domain and its organization into direct repeats of AGGTC[A/G] motifs are denoted by arrows. (C) Following exposure to RA for 2 days, F9 cells were transiently transfected with the vectors and exposed to RA as described in the legend of Table 1. On the left are the sequences of the β_1-AR promoter between −125 and −100. The mutations induced by site-directed mutagenesis of the promoter extending from −3311 to −100 are underlined. The arrow containing the X indicates a disrupted repeat. The data represent the percent expression of the β_1-AR constructs relative to the expression of the pGL3*control* plasmid in F9 cells. *Error* represents the ± SEM for the combined results of three transfections. Each transfection was performed in triplicate. Key: (*) $P < 0.05$.

the −3311 and −100 vector in these F9 cells was similar to the pre-exposed group (data not shown). Because the effect of RA on these cells was more robust in the pre-exposed group, subsequent luciferase activities were determined in F9 cells that were exposed to RA before and after DNA transfection.

In the next experiments, we determined whether the sequences between −125 and −100 could confer hormonal responsiveness in isolation from the rest of the promoter. These sequences were ligated into the *Nhe*I site in the pGL2*promoter* vector. The pGL2*promoter* vector contains the luciferase gene driven by the enhancerless SV40 pro-

TABLE 1. Effect of retinoic acid on the promoter activity of the rat β_1-AR gene in F9 teratocarcinoma cells

	Luciferase activity as percent of pGL3*control*			
	F9 cells		F9 cells pre-exposed to 100 nM RA	
β_1-AR-*Luc* vector	−RA	+RA	−RA	+RA
pGL3*basic*	2 ± 1	1 ± 1	2 ± 1	3 ± 1
−3311, −126	16 ± 3	19 ± 4	19 ± 3	24 ± 4
−3311, −100	4 ± 2	7 ± 3	5 ± 2	16 ± 3*

In the left side of the table, the sequences in the 5′-flanking region of the β_1-AR gene that were cloned into the multiple cloning site of the promoterless luciferase vector, pGL3*basic*, are indicated. One-half of the F9 cells were pre-exposed to 100 nM RA for 2 days, and then all of the cells were transiently transfected with 5 μg equivalent of pGL3*basic* and 2 μg pSVβ*gal* as described under "Materials and Methods." The cells were exposed to calcium phosphate precipitates for 18 hr, washed twice with phosphate-buffered saline, and then exposed to 100 nM RA for an additional 48 hr. The activity of pGL3*control* in F9 cells in the absence or presence of RA was 62 ± 9 and 53 ± 6 fg firefly luciferase/mg protein, respectively. The data represent the percent expression of the β_1-AR constructs relative to the expression of the pGL3*control* plasmid. *Error* represents the ± SEM for the combined results of at least three transfections. Each transfection was performed in triplicate.

* $P < 0.05$.

TABLE 2. Effect of the wild-type or mutated sequences between −125 and −100 on the activity and retinoic acid responsiveness of the SV40 promoter in F9 cells

β_1-AR-luciferase vector	−RA	Luciferase activity of F9 cell extract (pg/mg) +RA		Fold change
pGL2*promoter*		26 ± 4	13 ± 3	0.5
[*wt* −125, −100] in pGL2*promoter*		1 ± 1	6 ± 1*	6
[*wt* −100, −125] × 3 in pGL2*promoter*		3 ± 2	70 ± 5†	23
[*Mut* −105/−102] in pGL2*promoter*	[A]	1.5 ± 0.5	1.5 ± 0.5	1
[*Mut* −111/−108] in pGL2*promoter*	[B]	3 ± 0.5	12 ± 1*	4
[*Mut* −116/−113] in pGL2*promoter*	[C]	3 ± 1	2 ± 1	0.7
[*Mut* −102/−111] in pGL2*promoter*	[A, B]	1 ± 1	1 ± 1	1
[*Mut* [−105/−102] − [−116/−113]] in pGL2*promoter*	[A, C]	4 ± 1	3 ± 1	0.8
[*Mut* −102/−111] in pGL2*promoter*	[A, B, C]	4 ± 1	3 ± 1	0.8

The sequences in the region between −125 and −100 or the mutations in this sequence that are described in the legend of Fig. 1 were ligated into the multiple cloning site of pGL2*promoter*. These plasmids were transfected and exposed to RA as described in the legend of Table 1. The data represent the activity of these plasmids in picograms of firefly luciferase per milligram of protein. *Error* represents the ± SEM for the combined results of three transfections. Each transfection was performed in triplicate (N = 9).

* $P < 0.05$.

† $P < 0.001$.

moter and is used to test the transactivation capabilities of putative enhancer elements. Transcription of the pGL2*promoter* vector in F9 cells was not stimulated by RA. Either one or three copies of the sequence between −125 and −100 were ligated into the multiple cloning site of pGL2*promoter* (Table 2). Transcription from the vector harboring a single copy of the oligomer increased 6-fold in response to RA in endormal F9 cells, whereas transcription from the vector containing three copies of the oligomer was increased 23-fold in response to RA. These results indicate that the −125 to −100 element was responsive to RA.

Identifying Nucleotides Involved in RA Action by Site-Directed Mutagenesis

The next set of experiments was designed to identify the nucleotides between −125 and −100 required for inducing β_1-AR transcription by RA. There are three AGGTCA-like motifs on the bottom strand between nucleotides −125 and −100 (Fig. 1, A and B). To determine which of these repeats was involved in RA responsiveness, 4 bp mutations were introduced into the GGTC core of each motif in the context of the −3311 bp β_1-AR promoter. These vectors were transfected into F9, and the effect of RA on reporter gene activity was assessed.

Transcription from the wildtype β_1-AR-luciferase vector was increased 3-fold by adding RA in transient transfection experiments in F9 cells (Table 1, Fig. 1C). The β_1-AR-luciferase vector containing the mutation between nucleotides −116 and −113, in which domain C was disrupted, was unable to respond to RA (Fig. 1C). Likewise, disruption of domain A in the Mut −105 to −102 vector eliminated RA responsiveness. Mutating the sequences between −111 and −109 in domain B did not decrease the RA responsiveness of the β_1-AR promoter. Three broad mutations were also tested. Vectors containing mutations in either domains A and C, A and B, or A, B, and C were not induced by RA. These data indicate that the induction by RA was dependent on the repeats A and C and demonstrated that RA stimulated transcription through a DR5.

The next experiments examined whether the same domains in the β_1-AR-RARE were required for hormone responsiveness when the element was out of the context of the β_1-AR promoter. The oligomers representing the wild-type sequence between −125 and −100 and the mutations described in Fig. 1 were ligated into the multiple cloning site of pGL2*promoter* vector (Table 2). F9 cells were pre-exposed to 100 nM RA for 2 days and then transfected with the vectors described in Table 2 and exposed to RA or buffer for an additional 2 days. The luciferase vectors Mut −105, −102 and Mut −116, −113, in which the 5′-most and 3′-most repeats (domains A and C) were disrupted, respectively, were unable to respond to RA. However, RA stimulated by 4-fold transcription of the luciferase vector Mut −111 to −108, in which domain B was disrupted. These results confirm that domain B was not involved in the hormonal response. As in the intact gene, the broad mutations in domains A and B, A and C, or A, B, and C eliminated the RA response. These data confirm that the direct repeats contained within nucleotides −106 and −101 and −117 and −112 were required for RA-mediated stimulation of β_1-AR transcription.

Binding of the RARα to the Sequences between −125 and −100 in the β_1-AR Promoter

As the effects of RA are elicited by the binding of the hormone–receptor complex to response elements, the next experiments examined the interaction of RARα with the β_1-AR-RARE. Gel mobility assays were used to examine the binding of the RAR to the sequence between −125 and −100 (Fig. 2). To prepare the RARα, the RAR cDNA was placed in the pET 8C vector, and this vector was introduced into the *E. coli* BL21. Lysates were prepared from *E.*

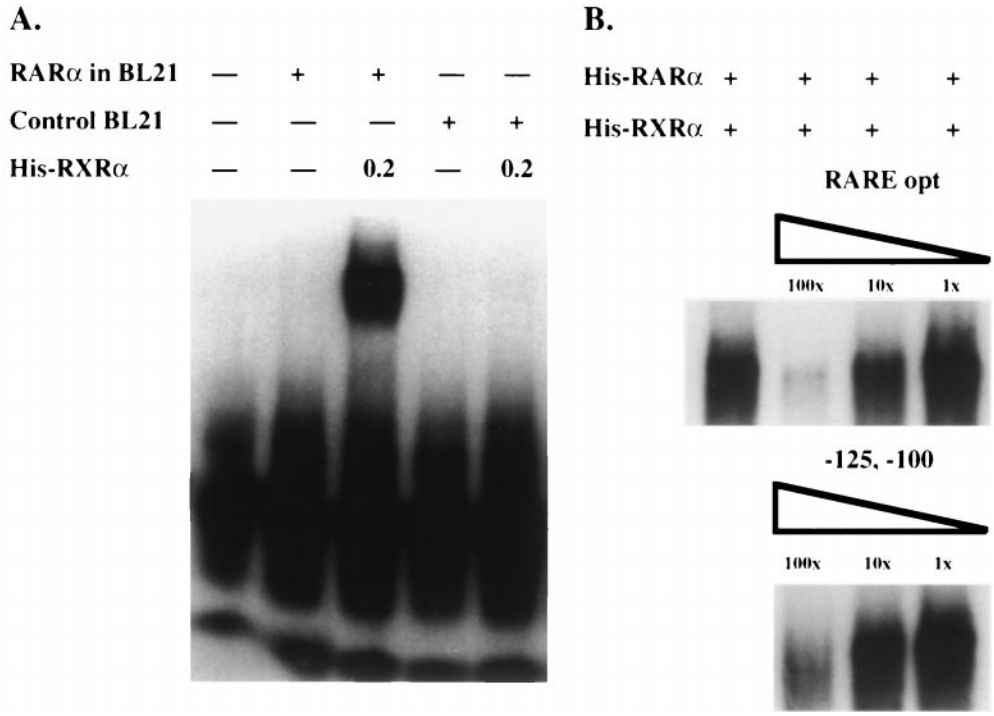


FIG. 2. Heterodimer formation between RARα and RXRα over the sequences between −125 and −100 in the β_1-AR gene. (A) The oligomer representing the sequence between −125 and −100 in the β_1-AR gene was labeled with [α^{32}P]dCTP. The labeled oligomer (10,000 cpm) was incubated with 1 μL extract of BL21 *E. coli* expressing the empty pET 8c vector or the vector harboring the human RARα cDNA or with BL21 extracts and 5 ng of histidine-tagged RXRα (His-RXRα) in binding buffer containing 1 μg poly[dI · dC] for 20 min at room temperature. (B) Histidine-tagged RARα (His-RARα) and His-RXRα were purified from *E. coli* by nickel affinity chromatography. Each binding reaction mixture contained 7000 cpm of ^{32}P-labeled oligomer representing the sequences between −125 and −100 in the β_1-AR promoter, 5 ng of His-RARα, 5 ng His-RXRα, and unlabeled competing oligomers. The competitor oligomers represent either the wild-type β_1-AR-RARE sequence (wt −125, −100) or the optimal RARE sequence in the RARα promoter. As indicated above each lane, a 100-fold excess (100×), 10-fold excess (10×), or an equal amount of unlabeled oligomer (1×) was added to each binding reaction mixture and incubated for 20 min at room temperature. The complexes were resolved on a 5% nondenaturing polyacrylamide gel in Tris/glycine and visualized by autoradiography.

coli BL21 containing either the empty pET 8C vector or the pET 8C vector containing the RARα cDNA. The lysate containing RARα did not bind to the ^{32}P-labeled oligomer containing the sequence from −125 to −100 (Fig. 2A). However, RARα did bind very efficiently when RXRα was added to the binding reaction. The lysate encoding the expressed pET 8C vector did not bind to the labeled oligomer even when 0.2 ng of histidine-tagged RXRα was added (Fig. 2A). These data indicate that RAR binds to the β_1-AR-RARE as a heterodimer with RXR. The complex between the ^{32}P-β_1-AR-RARE and RARα-RXRα heterodimers was disrupted by adding excess unlabeled oligomer representing the β_1-AR-RARE as well as by the optimal RARE, which is composed of direct repeats of AGGTCA hexamers that are separated by 5 bp (Fig. 2B).

The involvement of nuclear RAR and RXR in binding to the ^{32}P-labeled oligomer corresponding to the sequence between −125 and −100 was analyzed by adding an RARα antibody to the binding reaction (Fig. 3). In these experiments, neither RARα nor RXRα bound to the β_1-AR-RARE, but RARα-RXRα heterodimer bound very efficiently. The complexes between RARα and RXRα were supershifted by adding the anti-RARα IgG but not by the non-immune IgG (compare lanes 7 and 8 in Fig. 3). Neither the antibody nor the IgG alone formed any complexes with the β_1-AR-RARE. These data confirm that the RAR binds the β_1-AR-RARE as a heterodimer with RXR.

Identifying the RAR Binding Nucleotides in the Sequences between −125 and −100

Gel mobility assays were used to characterize further the interaction of RAR-RXR heterodimers with the sequences between −125 and −100 (Fig. 4). To determine which nucleotides are required for RAR-RXR binding to the sequence between −125 and −100 in the β_1-AR promoter, an experiment was conducted in which unlabeled oligomers were allowed to compete for the binding of RARα-RXRα heterodimers to the ^{32}P-labeled oligomer (Fig. 4). The competitor oligomers were either the wild-type sequence between −125 and −100 or oligomers containing the singly disrupted repeats shown in Fig. 1. The unlabeled wild-type oligomer and the oligomer with mutations in the sequence between −111 and −108 in domain B were the most effective competitors. The oligomers with a mutation in either domain A or domain C greatly reduced the ability

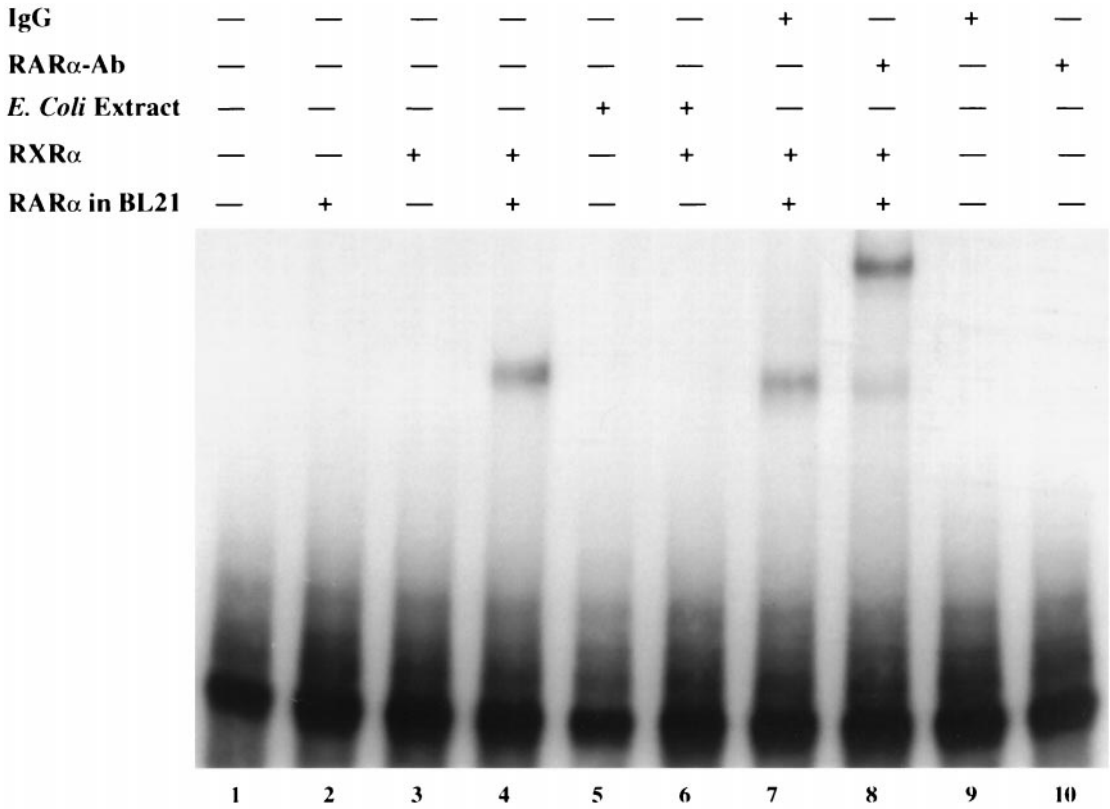


FIG. 3. Characterization of the binding of RARα-RXRα heterodimers to the sequences between −125 and −100 by gel super-shift assays. Gel mobility assays were conducted as described in Fig. 2. Each binding reaction contained 25,000 cpm of ^{32}P-labeled oligomer representing the sequence from −125 to −100 in the β_1-AR promoter and 1 μL extract of BL21 *E. coli* expressing the empty pET 8c vector or the vector harboring the RARα cDNA or His-RXRα as indicated by +. The anti-RARα IgG (RARα-Ab) or non-immune IgG (IgG) was added to the binding mixtures as shown. Binding reactions were conducted for 20 min at room temperature and resolved as described in the legend of Fig. 2.

of these oligomers to compete for binding to the RAR/RXR-oligomer complex (Fig. 4). These data indicate that the AGGTCA motifs A and C are required for high-affinity binding as well as for activating β_1-AR gene transcription in response to RA.

In another set of experiments, we examined the binding of nuclear extracts from F9 cells to the β_1-AR-RARE (Fig. 5). Since RA induces the differentiation of F9 cells, nuclear proteins were prepared from untreated and RA-treated F9 cells. As shown in Fig. 5, several complexes were formed between the β_1-AR-RARE and F9 cell nuclear extracts. No difference was observed in the binding pattern of nuclear extracts from RA-treated and untreated cells. As shown in Fig. 5, the complexes between the nuclear extracts prepared from F9 cells and the labeled oligomer migrated more slowly than RARα-RXRα heterodimers. The complex between the oligomer and RAR-RXR heterodimers was shifted by the anti-RAR IgG, while the F9 complex was not shifted by the anti-RARα IgG. These observations indicate that additional proteins in F9 nuclear extract can bind to this complex.

DISCUSSION

F9 teratocarcinoma cells progress from embryonic stem cells to primitive endodermal cells when these cells are exposed to RA alone. In the primitive endodermal state, the cells become flat, show typical endodermal morphology, and express the protease tissue plasminogen activator as well as components of the basal lamina [29]. A separate developmental program can be initiated if these cells are exposed to cyclic AMP elevating agents (such as dibutyryl cyclic AMP) in combination with RA. In this case, F9 cells assume a parietal endoderm-like phenotype of early mouse embryogenesis [7]. Primitive endodermal (RA-treated) F9 cells contain a 3-fold higher β-AR density than the embryonal phenotype. Moreover, RA-differentiated cells express twice as many β-AR as the parietal endodermal cells [9]. Based upon pharmacological characterization of the expressed receptors by radioligand binding techniques, it was determined that most of the increase in β-AR density occurred in the β_1-AR subpopulation, which increased by about 4-fold after 5 days of continuous exposure to RA [9]. Based upon these observations, we reasoned that the effect of RA on the expression of the β_1-AR gene in F9 cells might result from transcriptional coupling to differentiation-mediated events. If the effect of RA were due solely to an effect on gene transcription, then maximal activation of the β_1-AR-luciferase chimera in response to RA would occur within 48 hr as has been observed in numerous genes whose transcription is under the control of RA [30]. In our

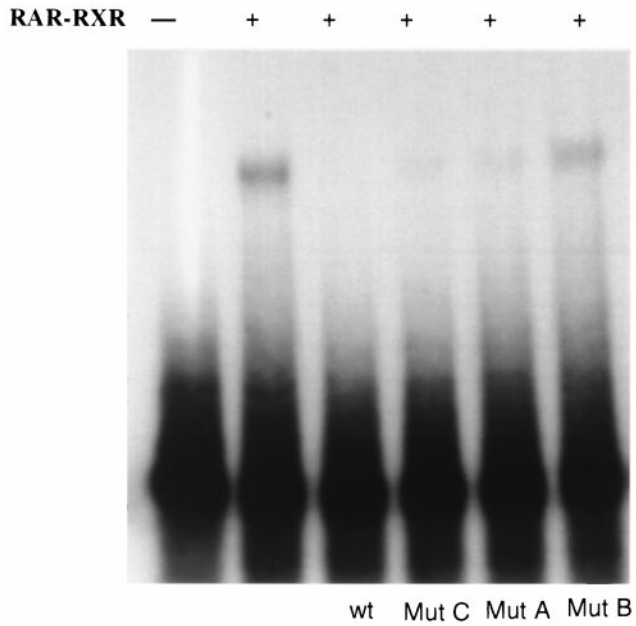


FIG. 4. Identification of nucleotides in the β_1-AR-RARE involved in binding RARα/RXRα heterodimers by oligonucleotide competition. Gel mobility assays were conducted as described in Fig. 2. Each binding reaction mixture contained 25,000 cpm of ^{32}P-labeled oligomer representing the sequences between −125 and −100 in the β_1-AR promoter and indicated set of proteins and unlabeled competing oligomers. The binding reactions contained a mixture of 1 μL of BL21 *E. coli* expressing the vector harboring the RARα cDNA, 5 ng of His-RXRα, and 100-fold molar excess of the indicated unlabeled competing oligomer. The competitor oligomers represent either the wild-type β_1-AR-TRE sequence (wt −125, −100) or the β_1-AR-TRE sequence (Mut) containing the base pair changes outlined in Fig. 1.

case, there was no difference in the activity of the −3311, −100-luciferase construct in cells that were exposed to RA or buffer for 2 days. Therefore, additional inputs appear to be required for the full effect of RA on β_1-AR gene transcription in F9 stem cells. Multiple changes occur following RA administration including a 20-fold increase in RARβ [31], up-regulation of *Hoxa-1* and the extracellular matrix protein laminin B1 and collagen IV genes [32], as well as cellular RA binding protein II, *Sparc* and *Rex-1* genes among others [33]. The time–course for some of these RA-associated events is interesting. For example, RARβ is maximally induced by 48 hr, whereas laminin B1 is not transcriptionally activated until 24–48 hr after RA addition and is not maximally induced until approximately 72 hr [31, 34]. The induction of these genes by RA appears to be mediated by specific subsets of RAR. For example, RARα is associated with RA-mediated induction of cellular RA binding protein II and *Hoxb-1* genes, while RARγ is associated with RA-inducible expression of *Hoxa-1*, *Hoxa-3*, laminin B1, collagen IV, *GATA-4* and *BMP-2* genes [35]. These findings provide evidence that the various subtypes of RAR have different but specific functions in the differentiation process of F9 cells and that RA actions are concentration- and time-dependent events. These considerations appear to be important for the effect of RA on β_1-AR transcription in F9 cells as revealed in the data of Table 1. It seems likely that RA administration increases the abundance of RAR so that after 48 hr RA can stimulate the expression of the β_1-AR-luciferase vectors.

The β_1-AR-RARE between nucleotides −125 and −100 is a complex element that mediates several important aspects of the regulation of β_1-AR gene expression. Previously, we have demonstrated that this element can suppress the expression of the rat β_1-AR gene through a domain between nucleotides −118 and −121 [16]. This effect is dependent upon sequences 2500-bp 5′ to the ATG [16]. Here we report that the element between −101 and −117 is involved in RA responsiveness of the β_1-AR promoter. We used several criteria to demonstrate the involvement of the −101 to −117 domain in RA-mediated effects on transcription. The data in Fig. 1 reveal that the sequences between −102 and −105 (domain A) and −113 and −116 (domain C) are essential for RA-induced activation of the 3-kb promoter of the β_1-AR gene. Similarly, domains A and C are required for the RA response in isolation from the rest of the promoter (Table 2). Our gel mobility assays indicated that the RARα isoform bound to the β_1-AR-RARE as a heterodimer with RXRα and that heterodimer binding could be competed by the optimal-RARE sequence in the RARα promoter as well as by the β_1-AR-RARE sequence between −100 and −126. Moreover, the data in Fig. 4 reveal that domains A and C are necessary for the binding of RAR-RXR heterodimers. Therefore, functional and biochemical assays imply that the sequences between −117 and −101 function as a *bona fide* RARE.

The involvement of a DR5 in the action of RA has been documented by Umesono *et al.* [22] and others who have shown that RA would stimulate through a DR5 as well as through direct repeats separated by 1 or 2 or 5 bp [18, 23, 36]. While the β_1-AR-RARE is composed of a usual DR5 motif, it is unusual, however, in its localization and specificity. First the β_1-AR-RARE is located 3′ to the transcriptional start sites of the β_1-AR gene, which are clustered around a region 253-bp 5′ to the translational initiation site [12]. The β_1-AR gene, unlike most genes, is intronless, and its basal transcription is regulated by domains 5′ and 3′ to the transcriptional start site. A 3′ domain between nucleotides −186 and −211 was identified by Searles *et al.* [12] as a major regulator of basal expression. In addition, the sequence between −121 and −118 suppresses the expression of this gene in the context of the 3 kb β_1-AR promoter [16]. Therefore, the RARE is localized in close proximity to domains involved in regulating the normal activity of the β_1-AR gene.

The β_1-AR-RARE is promiscuous in that it mediates responsiveness to thyroid hormones in ventricular myocytes from the same motifs that are involved in imparting RA responsiveness [37]. Hence, the region between −101 and −117 functions as a hormone-responsive motif in a cell-specific manner. The data in Fig. 5 support this tenet

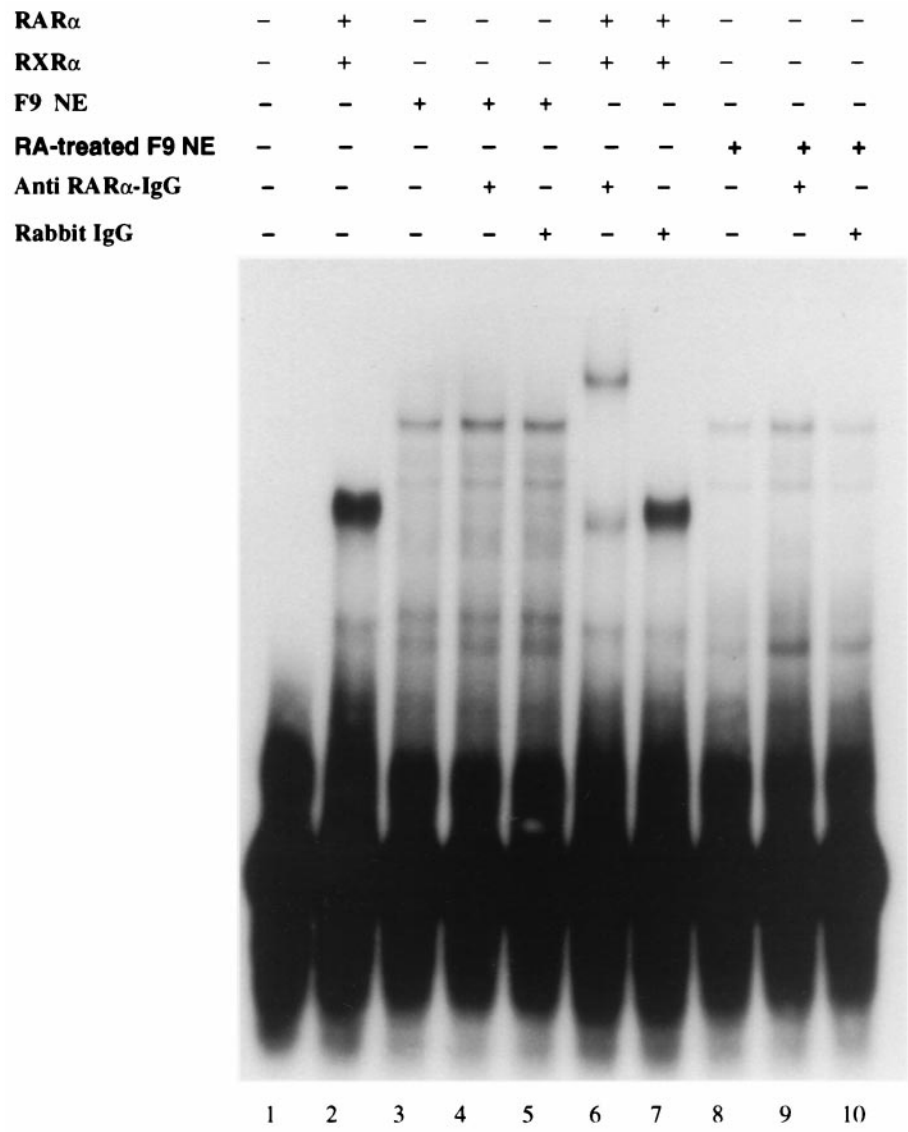


FIG. 5. Binding of nuclear proteins from F9 cells to the −125 to −100 element of the β_1-AR gene. Procedures for the gel mobility assay and the preparation of proteins from embryonal and RA-treated F9 nuclei extract (F9 NE) are described in "Materials and Methods." The binding reaction mixture contained 25,000 cpm of ^{32}P-labeled oligomer representing the nucleotides between −125 and −100 in the β_1-AR promoter, 1 μL of BL21 *E. coli* expressing the vector harboring the RARα cDNA, 5 ng of His-RXRα or 1 μL F9 nuclear extract from untreated (F9 NE) or 2 μL of nuclear extract from F9 cells treated with 100 nM RA for 5 days (RA-treated F9 NE) as indicated. The anti-RARα IgG or non-immune rabbit IgG was added to the binding mixtures as shown. Binding reactions were conducted for 20 min at room temperature and resolved as described in the legend of Fig. 2.

because the oligomer corresponding to the −125 to −100 sequence bound proteins from F9 nuclei that were distinct from RAR-RXR heterodimers. Likewise, nuclear extracts prepared from ventricular myocytes also showed that proteins other than the RAR would bind to this element (data not shown). The binding of thyroid hormone receptors and RAR to their respective element is independent of ligand [4]. Therefore, in the β_1-AR, whether the −101 to −117 element is functioning as a RARE or thyroid hormone-responsive element may reflect the abundance of RARs and thyroid hormone receptors, as well as other tissue-specific factors. In F9 cells, pretreatment with RA increases the abundance of RAR and possibly the necessary factor that may cause this element to be a functional RARE.

The interaction between RAR and thyroid hormone receptors and the activation of transcription from the same element in the β_1-AR promoter identify a new feature for hormonal transactivation of transcription. The involvement of overlapping nucleotides in imparting thyroid hormone and RA responsiveness is not unique to the β_1-AR. The PEPCK gene is induced by thyroid hormone and RA from sequences between nucleotides −319 and −335. Thyroid hormones stimulate PEPCK transcription through two direct repeats of the hexamer motif between nucleotides −330 and −319 [38]. Transactivation of PEPCK transcription by RA involves the region encompassing the nucleotides between −319 and −335 and utilizes one of the hexamers involved in mediating the effect of thyroid hormone on PEPCK transcription [39]. Therefore, in analogy with the β_1-AR, the activation of the PEPCK promoter by thyroid hormone and RA is mediated by nucleotides that are shared among the different transcription factors. Further studies will be required to identify the cell-specific factors that are involved in selective regulation of hormone responsiveness from a single element.

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