ELSEVIER

Molecular Brain Research 87 (2001) 204–213

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

A functional retinoic acid response element (RARE) is present within the distal promoter of the rat gonadotropin-releasing hormone (GnRH) gene

Sehyung Cho[a], Jean-Ju Chung[a], Youngshik Choe[a], Hueng-Sik Choi[b], Do Han Kim[c], Kunsoo Rhee[a], Kyungjin Kim[a,*]

[a]*School of Biological Sciences and Research Center for Cell Differentiation, Seoul National University, Seoul, 151-742, South Korea*
[b]*Hormone Research Center, Chonnam National University, Kwangju, 500-757, South Korea*
[c]*Department of Life Science, Kwangju Institute of Science and Technology, Kwangju, 500-712, South Korea*

Accepted 24 February 2000

Abstract

We previously demonstrated that all-*trans*-retinoic acid (all-*trans*-RA) regulates gonadotropin-releasing hormone (GnRH) release and gene expression in rat hypothalamic fragments and GT1-1 neuronal cells. Promoter analysis of rat GnRH gene revealed that the enhancing effect of all-*trans*-RA on GnRH transcription is mediated by *cis*-elements localized within −1640/−1438 of the rat GnRH promoter. In the present study, we attempted to localize functional retinoic acid response elements (RAREs) within the all-*trans*-RA-responsive region of the rat GnRH gene. Sequence analysis showed that there exist three putative repeats of AGGTCA-related sequences (−1637/−1617, −1579/−1562, and −1494/−1470) within this promoter sequence. Among them, only the −1494/−1470 sequence could compete the specific binding of GT1-1 nuclear extracts to the consensus RARE (direct repeat of AGGTCA with a 5-bp spacer, DR-5) and vice versa in electrophoretic mobility shift assays. In addition, like consensus RARE, the −1494/−1470 sequence could confer all-*trans*-RA responsiveness when inserted into the upstream region of SV40 promoter. Treatment of GT1-1 cells with all-*trans*- or 9-*cis*-RA increased the specific bindings of GT1-1 nuclear extracts to the consensus RARE and to the −1494/−1470 sequence while not affecting the specific binding to the cAMP response element (CRE). Both retinoids induced RARβ gene expression in GT1-1 cells. The −1494/−1470 sequence (5′-TCTT**A**G**G**A**C**TCTGTG**T**G**A**C**C**TAAGA) is similar to the direct repeat of T**G**AC**C**T (complementary sequence of A**G**GT**C**A) with a spacer of 5 bp (i.e. DR-5 in the reverse orientation). A mutation of the second core recognition motif of the −1494/−1470 sequence to a more divergent one from consensus RARE (from T**G**AC**C**T to T**T**AC**A**T) abolished the responsiveness to all-*trans*-RA, whereas a mutation of first core recognition motif to a more TGACCT-like sequence (from **A**G**G**ACT to **T**G**A**ACT) increased the responsiveness to all-*trans*-RA. These results indicate that the −1494/−1470 sequence is indeed a weak but functional RARE of the modified DR-5 type. Taken together, these data indicate that all-*trans*-RA enhances GnRH transcription via functional RARE present in the distal region of the GnRH promoter.

Theme: Endocrine and autonomic regulation

Topic: Hypothalamic-pituitary-gonadal regulation

Keywords: Gonadotropin-releasing hormone; All-*trans*-retinoic acid; GT1-1 cell; Transcriptional regulation; Retinoic acid response element

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a key neuroendocrine regulator controlling reproduction and development in vertebrates. The synthesis and secretion of GnRH is affected by a number of factors including neurotransmitters, peptides, steroids and neurotropic fac-

*Corresponding author. Present address: School of Biological Sciences, Seoul National University, Seoul, 151-742, South Korea. Tel.: +82-2-880-6694; fax: +82-2-872-6560.

E-mail address: kyungjin@snu.ac.kr (K. Kim).

0169-328X/01/$ – see front matter
PII: S0169-328X(01)00021-3

tors (reviewed in Refs. [19,26]). Recently, we reported evidence that all-*trans*-retinoic acid (all-*trans*-RA), an active metabolite of vitamin A, may function as another important regulator in GnRH neurons [9]. We demonstrated that GnRH neurons express functional retinoid receptors. In addition, we showed that all-*trans*-RA stimulates GnRH secretion and increases GnRH mRNA levels via enhanced gene transcription. The promoter region responsible for the all-*trans*-RA action was mapped within −1640/−1438 of the rat GnRH 5′-flanking sequence.

Vitamin A is an essential micronutrient, which plays critical roles in diverse biological processes such as vision, growth, reproduction, immunity, and epithelial tissue maintenance [1,5,7,43,46]. Although the importance of vitamin A in the maintenance of normal reproductive function has been known for a long time [52], the precise role of vitamin A in this process is only partially understood. Retinoids seem to be essential for spermatogenesis [16], oocyte maturation [50] and normal embryonic development [40]. Previous studies also indicated involvement of retinoids at the level of pituitary [2,21]. The regulation of GnRH gene expression and secretion by all-*trans*-RA expands the role of vitamin A to the hypothalamic level.

It is well documented that the biological functions of all-*trans*-RA are mediated by high affinity retinoic acid receptors (RARs) that act as ligand-activated transcription factors (reviewed in Refs. [5,17,30,33]). The RARs belong to the superfamily of steroid/thyroid/vitamin D/retinoid hormone receptors and there exist three distinct subtypes, α, β, and γ. When activated by their cognate ligand all-*trans*-RA, RARs bind as homodimers or as heterodimers with retinoid X receptors (RXRs) to DNA sequence elements of target genes known as retinoic acid response elements (RAREs). In this way, RARs either enhance or repress the transcription of target genes. Sequence analysis of the known RAREs of several genes characterized a conserved RARE sequence (reviewed in Ref. [18]). The conserved RARE is a direct repeat of nucleotide sequence AGGTCA (also known as half site or core recognition motif (CRM)) with spacing nucleotides of 5 bp (hence abbreviated as DR-5). The best-known example of a DR-5 type RARE is one that is found in the upstream promoter region of the RARβ gene [11]. However, examination of natural RAREs responding to all-*trans*-RA showed wide variations in sequence, orientation and spacing. For example, analysis of the medium acyl-CoA dehydrogenase gene has led to the identification of a complex RARE consisting of three CRMs [37]. The first two motifs are arranged as inverted palindromes spaced by 8 bp and the third motif is arranged as a direct repeat with respect to the second motif at a spacing of 1 bp. A similar RARE with two inverted palindromic RARE consensus sequences separated by an eight-nucleotide spacer was found in the second intron of the mouse H2Kb gene [22]. Moreover, it was reported that the human RAR can interact with a palindromic element located between −347 and −328 of the human immunodeficiency virus type 1 (HIV-1) long terminal repeat (LTR) [36]. Thus, it seems likely that not only DR-5 but also other arrangements of AGGTCA-related sequences can be a functional RARE depending on the promoter context.

In the present study, we attempted to identify functional RARE within the all-*trans*-RA responsive region of the rat GnRH promoter. Of three putative AGGTCA-related sequence repeats examined, we found that the −1494/−1470 sequence is a weak but functional RARE of DR-5 type.

2. Materials and methods

2.1. Materials

All-*trans*-RA was obtained from Sigma (St. Louis, MO, USA). Materials for cell culture were purchased from Gibco (NY, USA). Other chemicals, if not mentioned otherwise, were from Sigma. The plasmid pGnRH3.0LUC (kindly provided by J.L. Roberts, Mt. Sinai School of Medicine, USA) contains 3 kb of the 5′ flanking sequence of the rat GnRH promoter (−3002 to +88) fused to the luciferase reporter vector pXP2 [35]. pCMV-βgal which contains the cytomegaloviral promoter fused to the β-galactosidase gene was obtained from Stratagene (La Jolla, CA, USA). pGL3 luciferase reporter vectors were purchased from Promega (Madison, WI, USA).

2.2. Cell culture

GT1-1 cells (kindly provided by H. Jarry, University of Göttingen, Germany) were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin/streptomycin, and 10% fetal bovine serum (FBS) under a humidifying atmosphere containing 5% CO_2 at 37°C [9]. Medium was refreshed every 2–3 days. Prior to all-*trans*-RA treatment, cells were incubated at least 2 days in phenol red-free DMEM containing 10% dextran-coated charcoal-stripped FBS.

2.3. Reverse transcription-coupled polymerase chain reaction (RT-PCR)

RT-PCR was performed as described previously [44] with slight modifications. Briefly, 1 μg of total RNA was reverse-transcribed in the presence of random hexamer (50 pmol) and dNTP (1 mM) at 42°C for 1 h. Aliquots (usually 1 μl) of the RT mixture were subjected to PCR cycles as follows. Step 1 (initial denaturation): 95°C for 5 min, step 2 (amplification): 95°C for 1 min, 55°C (for RARα and β) or 50°C (for RARγ) for 1 min, 72°C for 1.5 min for 35 cycles; and step 3 (final extension): 72°C for 10 min. RAR subtype-specific PCR primers were designed to amplify

portions of cDNA sequences which encode the ligand binding domains of rat RARα (Hellemans et al., 1997, unpublished; EMBL accession No. AJ002940), mouse RARβ [20], and mouse RARγ [54]. The primer sequences are as follows: for RARα, upstream 5′-ATCGAGAAGGTGCGCAAAGC, downstream 5′-CAGCGTTGTGCATCTGAGTC to make 340-bp PCR products; for RARβ, upstream 5′-tatgaattc**AATGCTGGCTTCGGTCCTCT**, downstream 5′-tatggatcc**GGGAATGTCTGCAACAGCTGGA** to generate 502-bp PCR products; for RARγ, upstream 5′-CCCCTGGAGATGGATGACA, downstream 5′-GCGGTATCTGGGAAATGGTC to make 478-bp PCR products. All PCR products were cloned into the pGEM-T easy vector (Promega) and sequenced to confirm correct amplification.

2.4. Oligonucleotides and generation of heterologous promoter reporter vectors

Single-stranded oligonucleotides were synthesized from Bioneer (Cheongwon, Chungbuk, Korea). Oligonucleotide sequences are listed in Table 1. Single-stranded oligonucleotides were annealed to their complementary strands by heating to 85°C for 2–3 min and cooling slowly to below 40°C for several hours in the annealing buffer (10 mM Tris, pH 8.0, 0.1 mM EDTA, 200 mM NaCl). Complete annealing was confirmed by polyacrylamide gel electrophoresis. Otherwise, annealed oligonucleotides were eluted from polyacrylamide gels. Annealed oligonucleotides were used directly as probes for electrophoretic mobility shift assays and were also used to insert these putative elements into the SV40 promoter-driven luciferase reporter vector pGL3-promoter (Promega) at the NheI/XhoI sites. Correct insertions were confirmed by dideoxy sequencing.

2.5. Transfection experiment

GT1-1 cells were plated in 60-mm dishes and grown to 30–40% confluence for 2–3 days. The cells were washed twice with Dulbecco's phosphate-buffered saline (D-PBS) and the medium was changed to phenol red-free DMEM with 10% dextran-coated charcoal-stripped FBS. Then, 2 days later, the cells were washed twice with D-PBS and cultured in 5 ml of fresh medium for 2 h before transfection. The reporter plasmids for the transfection experiments were purified on Qiagen columns (Qiagen, Hilden, Germany) according to manufacturer's instructions and resuspended in 0.1× TE buffer (1 mM Tris, pH 8.0, 0.1 mM EDTA) at a concentration of 0.5–1 mg/ml. The DNA-calcium phosphate mixture was prepared by mixing one part of 2× BES-buffered saline (280 mM NaCl, 1.5 mM Na_2HPO_4, 50 mM BES, pH 6.96) with one part of DNA-calcium chloride solution (60 μg/ml DNA, 250 mM $CaCl_2$) and incubated at room temperature for 15 min before transfection. Each dish received 0.5 ml of the DNA-calcium phosphate mixture containing 5 μg luciferase reporter vector (pGnRH3.0LUC or 3XβRARE-LUC) and 10 μg β-galactosidase reporter vector (pCMV-βgal) dropwise and was incubated at 37°C under 5% CO_2 tension for 24 h. Excess DNA-calcium phosphate precipitates were washed out by rinsing twice with calcium- and magnesium-free D-PBS. Then the cells were cultivated in medium containing appropriate drugs for additional 24–48 h. Cell lysates were prepared by incubating cells in 0.4 ml of 1× reporter lysis buffer (Promega, Madison, WI, USA) for 15 min at room temperature. After centrifugation, supernatants were kept at −70°C until assayed. Protein concentration was measured with a protein assay kit (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard. Beta-galactosidase and luciferase assays were

Table 1
Oligonucleotide sequences[a]

Name	Sequence	Reference
−1637/−1617	CTAG**AGGTCTTGGGTAA**AT**TGGCCT** **TCCAGAACCCATT**TA**ACCGGA**AGCT	[25]
−1579/−1562	CTAGAAA**GCCTCA**AAA**GCCTCA** TTT**CGGAGT**TTT**CGGAGT**AGCT	[25]
−1494/−1470	CTAGTCTT**AGGACT**CTGTGT**GACCT**AAGA AGAA**TCCTGA**GACAC**ACTGGA**TTCTAGCT	[25]
DR-3 (VD_3RE)	CTAGAAA**AGGTCA**AAA**AGGTCA** TTT**TCCAGT**TTT**TCCAGT**AGCT	[18]
DR-4 (T_3RE)	CTAG**AGGTCA**CCGA**AGGTCA** **TCCAGT**GGCT**TCCAGT**AGCT	[18]
DR-5 (RARE)	CTAG**AGGTCA**CCGAAA**AGGTCA** **TCCAGT**GGCTT**TCCAGT**AGCT	[18]
PAL-5	CTAG**AGGTCA**CCGAA**TGACCT** **TCCAGT**GGCTT**ACTGGA**AGCT	
CRE	CTAGTGATTGCC**TGACGTCA**GAGAGCA ACTAACGG**ACTGCAGT**CTCTCGTAGCT	[39]

[a] All oligonucleotide pairs were synthesized to make NheI (CTAG) and XhoI (AGCT) compatible ends after annealing. ^{32}P-labeled probes were generated during the end-filling reaction using the Klenow fragment. Annealed oligonucleotides were also used to insert the putative elements into the reporter vector pGL3-promoter at the NheI/XhoI sites for use in transient transfection assays. Bold characters are putative or conserved CRMs.

performed using commercial enzyme assay kits (Promega). Untransfected GT1-1 cell extracts showed no background luciferase activity, and endogenous β-galactosidase activity was negligible under the assay condition used. Cytomegaloviral promoter-driven β-galactosidase activity was used to compensate the transfection efficiency.

2.6. Preparation of nuclear extracts

Nuclear extracts were prepared with a slight modification of the rapid nuclear extraction protocol described by Dignam et al. [12]. Cells were grown to 40–50% confluence in a 100-mm dish, and were fed with phenol red-free DMEM with 10% dextran-coated charcoal-stripped FBS and further incubated for 36 h. Cells were then treated with vehicle (0.1% ethanol), 1 μM all-*trans*-RA or 1 μM 9-*cis*-RA for 12–48 h. Cells were washed twice with D-PBS and harvested. Cell pellets were resuspended in 0.4 ml of buffer A (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF) and incubated on ice for 10 min. After vortexing for 10 s, nuclear pellets were obtained by two-step centrifugation at 2000 rpm for 40 s and at 12,000 rpm for 5 min. The resulting pellets were resuspended in 100 μl of buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 1 μg/ml of the protease inhibitors leupeptin, aprotinin, and pepstatin) and incubated on ice for 20 min. The nuclear extracts were centrifuged for 5 min, and the supernatant was aliquoted and stored at −70°C. Protein concentration was determined using the Bio-Rad protein assay system (Bio-Rad, Hercules, CA, USA).

2.7. Electrophoretic mobility shift assays (EMSA)

For radiolabeling, 4 pmol of annealed oligonucleotides were labeled with 20 μCi of [α-^{32}P]dCTP (Amersham) using Klenow fragment (Promega). Labeled probes were diluted to 40 fmol/μl. A total of 40 fmol of ^{32}P-probe were incubated with 2–5 μg of GT1-1 nuclear extracts in gel shift assay buffer (20 mM Hepes, pH 7.6, 0.1 mM KCl, 10% glycerol, 0.1% Nonidet P-40, 1 mM DTT) containing 2.5 μg of polydeoxyinosinic-deoxycytidylic acid in a 20-μl reaction. Binding reactions were performed on ice for 20 min and at room temperature for 30 min. The reaction mixture was run on a 5% native polyacrylamide (29:1) gel in 0.5× TBE (45 mM Tris-borate, 1 mM EDTA) at 150 V for 2 h to resolve protein-DNA complexes. For competition assays, a molar excess (usually 50-fold) of unlabeled double-stranded competitor DNA was added after addition of ^{32}P-labeled probes.

2.8. Statistical analysis

Data for GnRH promoter activity were statistically evaluated using Student's *t*-test or one-way analysis of variance followed by Fisher's least significant difference test for a post hoc comparison. Statistical significance was set at $P<0.05$.

3. Results

3.1. A distal promoter sequence (−1494/−1470) of the rat GnRH gene exhibits similar nuclear extract binding characteristics comparable to the consensus RARE

We have recently demonstrated that all-*trans*-RA enhanced GnRH transcription via distal promoter element(s) localized within −1640/−1438 of the rat GnRH 5′-flanking sequence [9]. In the present study, we attempted to localize functional retinoic acid response elements (RAREs) within this region. It is well established that direct repeats (DRs) of AGGTCA (or TGACCT in the reverse orientation) or closely related sequences with varying numbers of spacing nucleotides respond to all-*trans*-RA [18]. Thus, we searched repeats of AGGTCA (TGACCT in the reverse orientation) or related sequences within this region. Sequence analysis revealed the existence of three repeats of AGGTCA-related sequences within −1640/−1438 of the rat GnRH gene (Table 1). First, at −1637/−1617, a direct repeat of two putative CRMs with a spacer of 1 bp is followed by a third putative CRM which is arranged as a palindrome with respect to the second motif at a spacing of 2 bp. Second, at −1579/−1562, a direct repeat of GCCTCA with a spacer of 3 bp is present. Finally, at −1494/−1470, a palindromic arrangement of AGGTCA-related sequence with a spacer of 5 bp exists.

We performed cross-competition experiments to examine whether these putative enhancer elements have a binding affinity for nuclear proteins comparable to the consensus RARE. It is well established that direct repeats of AGGTCA with spacers of 3, 4, and 5 bp (i.e. DR-3, DR-4 and DR-5) are vitamin D_3 response element (VD_3RE), T_3 response element (T_3RE) and RARE, respectively [48]. Of these three putative enhancer elements of the GnRH promoter, EMSA showed that only the −1494/−1470 fragment showed qualitatively identical binding characteristics with DR-5 (Fig. 1). As shown in Fig. 1B, binding of GT1-1 nuclear proteins to −1494/−1470 sequence was strongly competed with DR-5 and DR-3. Competition with DR-4 was much weaker than with DR-5 or DR-3. In addition, the −1494/−1470 sequence could compete the binding of GT1-1 nuclear extracts to the DR-5 probe (Fig. 1A). The cAMP response element (CRE), used as a negative control, did not affect interactions of nuclear factors with the DR-5 or −1494/−1470 sequence and the opposite was also the case (Fig. 1C). This indicates that the distal sequence −1494/−1470 has

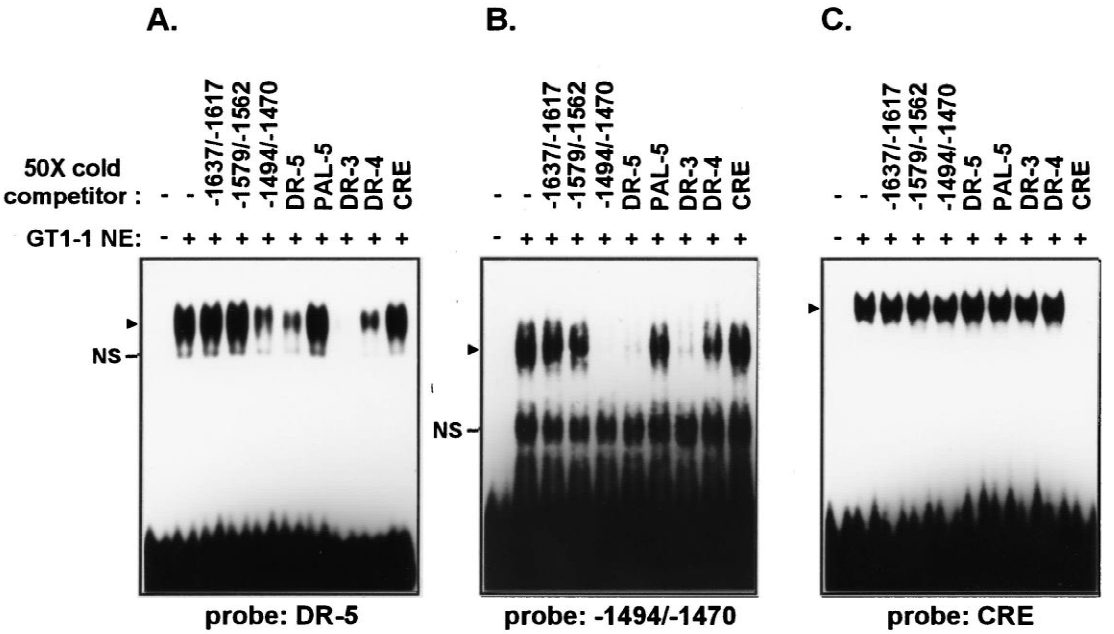

Fig. 1. The −1494/−1470 sequence of the rat GnRH promoter showed similar nuclear protein-binding characteristics comparable to the consensus RARE. Oligonucleotides corresponding to the consensus RARE (DR-5, panel A), −1494/−1470 sequence of the rat GnRH promoter (panel B) and consensus cyclic AMP response element (CRE, panel C) as described in Table 1 were annealed and radiolabeled with [α-^{32}P]dCTP during an end-filling reaction using Klenow fragment. About 40 fmol of each ^{32}P-labeled probes were incubated with 2 μg of GT1-1 nuclear extracts in the presence or absence of 50-fold molar excess (2 pmol) of cold competitors as indicated. Representative autoradiograms are shown here. These experiments were repeated at least three times with similar results.

identical binding characteristics with consensus RARE in vitro.

3.2. The −1494/−1470 sequence confers all-trans-RA responsiveness when inserted into a heterologous reporter vector

To test whether the −1494/−1470 sequence is indeed a functional RARE, we inserted these putative responsive elements or consensus response elements into the NheI/XhoI sites of the luciferase reporter vector pGL3, and transfected these heterologous reporter constructs into GT1-1 cells (Fig. 2). The pGL3-promoter vector itself did not respond to all-*trans*-RA. An insertion of a single copy of the consensus RARE (DR-5) conferred all-*trans*-RA responsiveness by inducing luciferase activity by ~3-fold. The heterologous promoters with DR-3, DR-4, PAL-5 (palindromic arrangement of AGGTCA with a spacer of 5 bp) or CRE did not respond to the all-*trans*-RA treatment. Among the three putative response elements of the GnRH promoter, only the −1494/−1470 sequence could confer all-*trans*-RA responsiveness. As shown in Fig. 2, the −1494/−1470 construct significantly induced luciferase activity by 70% over vehicle-treated control, while the other constructs failed to show any effect. These results indicate that the −1494/−1470 sequence is indeed a functional RARE.

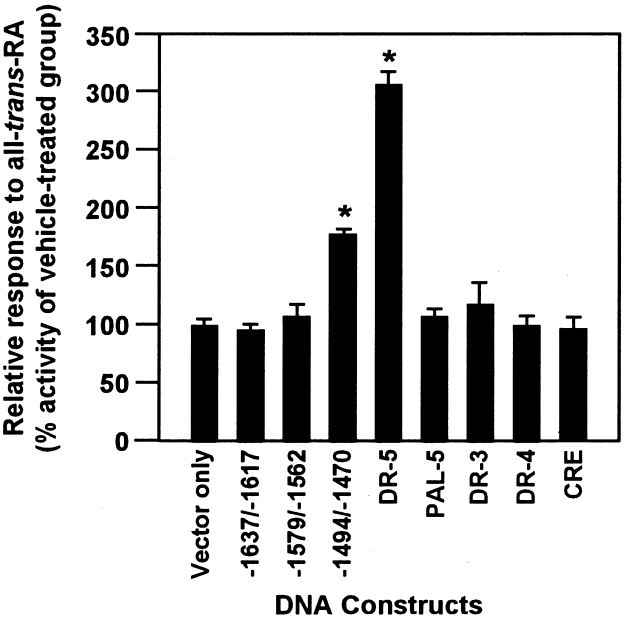

Fig. 2. The −1494/−1470 sequence of the rat promoter conferred all-*trans*-RA responsiveness when inserted into a heterologous reporter vector. Putative and consensus response elements described in Table 1 were inserted into the NheI/XhoI site of the pGL3-promoter vector. Correct insertions were confirmed by dideoxy sequencing. GT1-1 cells were transfected with each DNA construct and treated with all-*trans*-RA for 24 h. Luciferase activity in cell extracts was measured using a luminometer. *$P<0.05$ versus vehicle-treated control.

3.3. Retinoid treatment increases the binding activities to the −1494/−1470 sequence and induces RARβ expression

GT1-1 cells were treated with vehicle, all-*trans*-RA or 9-*cis*-RA (1 μM each) for 24 h and nuclear extracts were prepared to test whether retinoid treatment can alter consensus and putative response elements binding activities. Changes in the response elements binding activities were examined by EMSA. As shown in Fig. 3, binding activities to consensus RARE were significantly increased by both retinoids. In addition, treatment of GT1-1 cells with all-*trans*-RA increased the binding to the −1494/−1470 sequence, whereas it did not alter the binding activities to the −1637/−1617 and −1579/−1562 fragments (Fig. 3 and data not shown). This suggests a possibility that retinoid treatment alters gene expression of retinoid receptors. To test this, we examined changes in mRNA levels of the RAR genes. GT1-1 cells were treated with vehicle, all-*trans*-RA, or 9-*cis*-RA (1 μM each) and the total RNA was isolated at 24 and 48 h after the treatment. The mRNA levels of RAR subtypes were determined by RT-PCR procedure using subtype-specific primers (Fig. 4). RARα mRNA level was induced upon 24 h incubation with retinoids and remained high at 48 h. RARβ mRNA level was undetectable in vehicle-treated GT1-1 cells, but was induced by both retinoids. As expected, all-*trans*-RA was more active than 9-*cis*-RA in the induction of RARβ in GT1-1 cells. RARγ mRNA level was transiently induced 24 h after retinoid treatment and returned to the control level by 48 h.

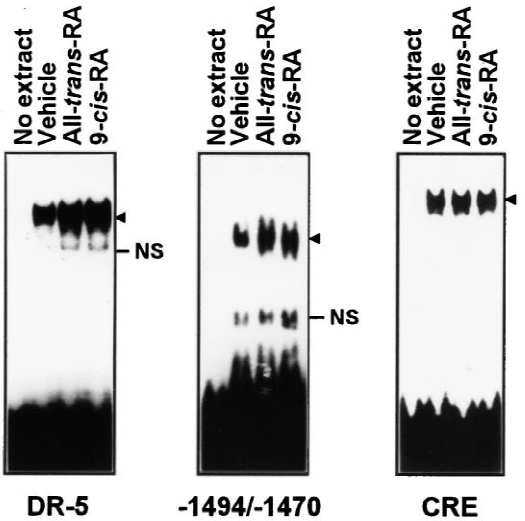

Fig. 3. Changes of binding activities to consensus nuclear hormone receptor response element following retinoid treatment. Oligonucleotides corresponding to DR-5, −1494/−1470 of the rat GnRH gene, and consensus CRE (Table 1) were annealed and radiolabeled with [α-^{32}P]dCTP during an end-filling reaction using the Klenow fragment. About 40 fmol of ^{32}P-labeled probes were incubated with 2 μg of GT1-1 nuclear extracts prepared from cells treated with vehicle, all-*trans*-RA (1 μM) or 9-*cis*-RA (1 μM) for 24 h. The binding reaction was performed as described in Fig. 1. These experiments were repeated at least twice with similar results.

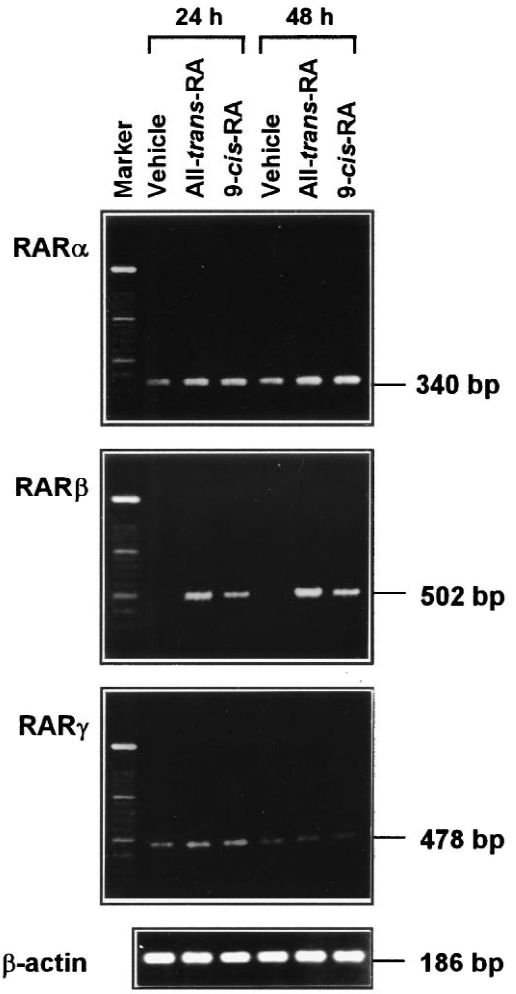

Fig. 4. RT-PCR analysis of RAR subtype gene expression in GT1-1 cells following retinoid treatment. Total RNAs were isolated from GT1-1 cells treated with vehicle, all-*trans*-RA (1 μM) or 9-*cis*-RA (1 μM) for 24 or 48 h as indicated. RT-PCR analysis was performed using RAR subtype-specific primers as described in Materials and methods. Representative pictures are shown here. RT-PCR analysis was repeated at least three times with similar results.

3.4. Point mutations in the −1494/−1470 sequence can alter responsiveness to all-trans-RA

It is well known that the consensus sequence of the 6-bp CRM for retinoid receptors is 5′-AGGTCA or more loosely defined as 5′-PuG(G/T)TCA [5] and can be read as 5′-TGACCT or more loosely 5′-TGA(C/A)CPy in the reverse orientation. A closer evaluation of the −1494/−1470 sequence of 5′-TCTT**AGGACT**CTGTG**TGAC-CT**AAGA reveals two CRMs separated by five nucleotides. The second CRM (5′-TGACCT) matches the consensus sequence exactly in the reverse orientation, while the first CRM is varied to 5′-AGGACT. The first motif

5′-AGGACT can be read as a variation of 5′-AGGTCA which has incorrect bases at the fourth and sixth positions. In this case, the −1494/−1470 sequence can be a variation of palindrome with a 5-bp spacer (PAL-5). Alternatively, the 5′-AGGACT sequence can be read as a variation of 5′-TGA(C/A)CPy where the first and third nucleotides are not matched. In this case, the −1494/−1470 sequence can be a variant of DR-5 in the reverse orientation. In any case, the second and fifth nucleotides of the first CRM that are known to interact directly with retinoid receptors are well conserved as a putative CRM. Alignment of the −1494/−1470 sequence with homologous sequences of either the mouse or human GnRH gene revealed two important features (Fig. 5A). First, the sequence of the second CRM and surrounding nucleotides is well conserved among these species. Second, the sequence of the first CRM has wide variations but the first nucleotide is usually thymidine (T) and more related to 5′-TGA(C/A)CPy rather than 5′-AGGTCA.

We designed point mutated oligonucleotides to evaluate whether the −1494/−1470 sequence is a direct repeat or a palindromic repeat (Fig. 5A). We examined changes in their ability to compete consensus RARE (Fig. 5B) and to confer all-*trans*-RA responsiveness (Fig. 6). A mutation of the second CRM to a more divergent one from the consensus sequence (from TGACCT to TTACAT, the G13T/C16A construct) completely abolished the ability to

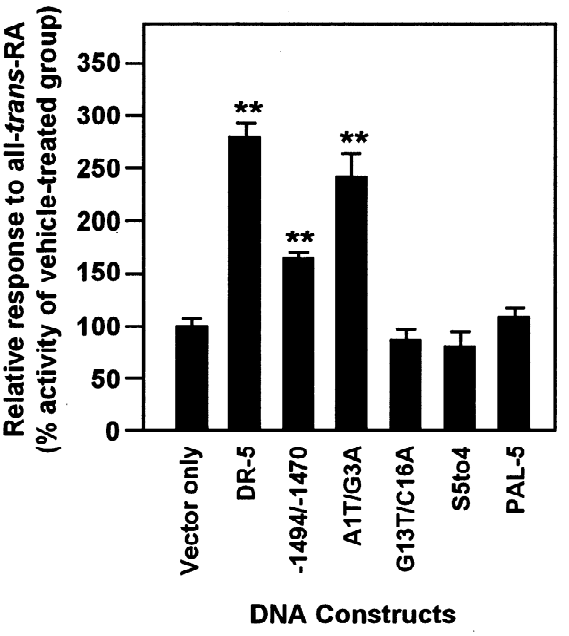

Fig. 6. A1T/G3A construct showed RARE activities comparable to the consensus RARE. Consensus or mutated sequences of the −1494/−1470 sequence were inserted into the NheI/XhoI sites of the pGL3-promoter vector. GT1-1 cells were transfected with each construct and treated with either vehicle or all-*trans*-RA (1 μM) for 24 h and analyzed for luciferase activity. These experiments were repeated twice with different DNA preparations with similar results (n=4).

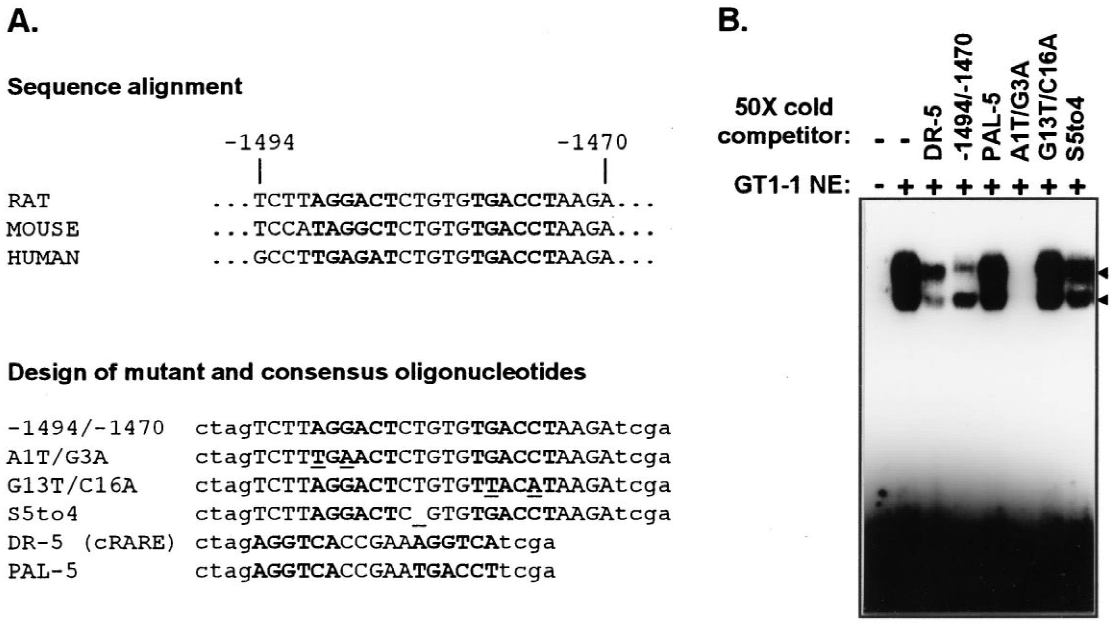

Fig. 5. Point mutation experiments. (A) Sequence alignment of the putative RARE sequence of the rat, mouse, and human GnRH gene (upper) and design of mutant oligonucleotides (lower). The putative RARE sequence of the rat GnRH gene (−1494 to −1470) was aligned with the homologous region of the mouse and human GnRH promoter sequence. To analyze the characteristics of the putative RARE sequence, the −1494/−1470 sequence was mutated as indicated (underlined). (B) The mutated oligonucleotides as indicated in (A) were annealed and radiolabeled with [α-^{32}P]dCTP during an end-filling reaction using the Klenow fragment. Electrophoretic mobility shift assays were performed as described in Fig. 1. Representative autoradiograms are shown here. These experiments were repeated three times with similar results.

compete consensus RARE and to confer all-*trans*-RA responsiveness (Fig. 5B). This indicates that the sequence of the second CRM is important for RARE activity. Moreover, a mutation of the first CRM to a more TGACCT-like sequence (from AGGACT to TGAACT, the A1T/G3A construct) increased the trans-activation response as well as the ability to compete the DR-5 binding. These results support the possibility that the −1494/−1470 sequence is indeed a type of direct repeat. In support of this notion, a consensus palindromic repeat of AGGTCA with a 5-bp spacer could not compete the consensus RARE and did not confer all-*trans*-RA responsiveness to a heterologous vector. Reducing the number of spacing nucleotides from five to four (the S5to4 construct) also completely abolished the characteristic of RARE.

4. Discussion

Various signals are known to regulate GnRH gene expression at the level of transcription (reviewed in Ref. [34]). POU-homeodomain transcription factors such as Oct-1 [6,10,14] and SCIP/Oct-6/Tst-1 [51], second messengers including phorbol ester [4,13,47,53], and feedback inputs to the GnRH neurons [8,23,24,27] contribute to the regulation of GnRH expression at the level of transcription. However, the physiological relevance of direct genomic actions of steroids on GnRH transcription in vivo is still obscured, since it has been impossible to detect the steroid receptors in GnRH neurons [15,45], which would specifically transduce the steroidal feedback inputs [3]. The presence of retinoid receptors in GnRH neurons [9] and the localization of functional RARE in the upstream of GnRH promoter provided in this study clearly indicate that RA is indeed a physiologically relevant signal to regulate GnRH neuronal activity.

Previously, we have showed that GT1-1 neuronal cells as well as hypothalamic tissues express functional retinoid receptor genes including RARα, RARγ, RXRα and RXRβ [9]. In addition, we have shown in the present study that RARβ expression can be induced in GT1-1 cells with retinoid treatment (Fig. 4). This strongly suggests that retinoids can be important regulators of GnRH neurons. Since retinoids are versatile bioactive molecules that are important for various biological processes including development, differentiation, vision, immunity and reproduction (reviewed in Refs. [5,7,43,46]), the specific functions of retinoids in GnRH neurons may also be multifaceted. However, it seems evident in the present study that one of the retinoid actions in GnRH neurons is regulation of GnRH transcription as dictated by the presence of functional RARE in the distal region of the GnRH promoter. Other possible actions of retinoids on development, migration and differentiation of GnRH neurons await future experiments.

Several lines of evidence suggest that the −1494/−1470 sequence is indeed a functional RARE of modified DR-5 type. First, the mouse and human GnRH promoter sequences homologous to the −1494/−1470 sequence are closer to direct repeats rather than palindromes (Fig. 5A). In addition, the −1494/−1470 sequence is composed of two CRMs that are separated by 5 bp. The second CRM (5′-TGACCT) matches the consensus sequence exactly in the reverse orientation, while the first CRM (5′-AGGACT) has two mismatches compared to the consensus motif of 5′-TGA(C/A)CPy at position 1 and 3. Nevertheless, the second base of guanine (G) and the fifth base of cytidine (C) of the first CRM are well conserved. These bases are highly conserved among naturally occurring response elements and were reported to be contacted directly by Lys22 and Arg27 of the nuclear receptor DNA binding domain (DBD), respectively [28,42]. Thus, these two bases of the first CRM of the −1494/−1470 sequence may ensure the binding of retinoid receptors, as demonstrated by specific competition of the binding of GT1-1 nuclear extracts to the DR-5 and vice versa. In addition, this may allow all-*trans*-RA responsiveness when inserted into a heterologous promoter vector. Likewise, a mutation of the well-conserved second CRM to a divergent one at position 2 and 5 of the CRM (the G13T/C16A construct) completely abolished the ability to compete DR-5 binding as well as the trans-activation activity. Thus, the second CRM seems indispensable for the maintenance of RARE activity.

The weak induction activity of the −1494/−1470 sequence by all-*trans*-RA compared to consensus RARE can be attributed to the two mismatches in the first CRM, since the mutation of the two bases of the first CRM to a more conservative one (the A1T/G3A construct) recovered the full trans-activation activity (Fig. 6) and enhanced competition with the consensus RARE (Fig. 5B). This also raises a possibility that, not only the bases in direct contact of the receptor DBD but also other surrounding bases of the CRM are important for high affinity binding and/or full trans-activation activity (see Ref. [31] and references therein).

The 5-bp spacing between the two CRMs of the −1494/−1470 sequence is also important for the RARE activity. Indeed, it is well established that the nucleotide spacing between direct repeats of CRMs determines the specificity of binding among members of the nuclear receptor superfamily. Preferential transcriptional responses to vitamin D_3, thyroid hormone (T_3) and all-*trans*-RA were observed on direct repeat elements spaced by three (DR-3), four (DR-4) and five (DR-5) base pairs, respectively [48]. Subsequent numerous studies established the ‘1 to 5 rule’, where the DR-1 was identified as an RXR response element (RXRE) or peroxisome proliferator response element (PPRE) and the DR-2 as a second type of RARE (reviewed in Refs. [18,29,32]). In accordance with this rule, a reduction of spacing from five to four nucleotides (the S5to4 construct) completely abolishes the transactiva-

tion activity (Fig. 6), supporting once again that the −1494/−1470 is indeed a DR-5 type RARE. The reduction of spacing nucleotides however, slightly decreased the ability to compete with DR-5 binding in vitro. This is not surprising, since DR-3, DR-4 and DR-5 share common CRMs and can compete with each other in EMSA under appropriate binding conditions [48]. Moreover, these conserved response elements have a common heterodimeric partner RXR in their high affinity DNA-protein complexes (reviewed in Ref. [41]).

It should be mentioned here that, when we examined the two other sequence elements of the rat GnRH promoter (the −1637/−1617 and −1579/−1562) in terms of RARE activity, neither of them could compete with consensus RARE in in vitro binding and vice versa (Fig. 1 and data not shown). Moreover, these sequences did not respond to all-*trans*-RA in the heterologous promoter analysis. Although specific DNA-protein complexes formed with GT1-1 nuclear extracts were detected by EMSA, these two sequences are unlikely to be related to retinoid signaling. Indeed, the sequence, spacing and orientation of the putative CRMs of these two sequences examined in the present study are more divergent from the conserved CRM (Table 1) and from other known natural RAREs.

In the present study, treatment of GT1-1 cells with all-*trans*-RA increased the binding of GT1-1 nuclear extracts to the consensus RARE and to the −1494/−1470 sequence. Since the in vitro binding of the nuclear receptors to the DNA elements as measured by EMSA is independent of the presence of their cognate ligands (reviewed in Ref. [38]), we assumed that increases in the specific binding could be attributed to enhanced RAR subtype gene expression. Accordingly, we observed that RAR subtype gene expression was induced by retinoid treatment in GT1-1 cells (Fig. 4). Constitutively expressed RARα and RARγ transcripts were significantly increased upon treatment of retinoids. Furthermore, the RARβ subtype, which was undetectable in vehicle-treated GT1-1 cells, was strongly induced upon retinoid treatment. This may be due to the presence of a strong RARE in the upstream sequence of the RARβ promoter [11]. Although changes in retinoid receptor transcripts and indirect evidence for changes in the protein levels of RARs were provided by this study, the retinoid signaling cascade in GT1-1 cells leading to enhanced GnRH transcription remains to be clarified. Specifically, the subtype(s) of RAR that are responsible for the enhancement of GnRH expression should be determined either by subtype selective retinoids or by RAR co-transfection studies. In addition, this study could not exclude the possibility that cell-specific expression of cofactors may contribute to the DNA binding activity of retinoid receptors in a ligand-independent manner as has been studied recently (reviewed in Ref. [49]).

In conclusion, the present study clearly demonstrated that all-*trans*-RA activates GnRH transcription via a functional DR-5 type RARE present at −1494/−1470 of the GnRH promoter.

Acknowledgements

We would like to thank John Blackford (NIH) for English correction. This work was supported by grants from the Ministry of Education through Genetic Engineering Research Program and from the Ministry of Science and Technology through the Brain Science Research Program. S. Cho, J.-J. Chung and Y. Choe are supported by the Brain Korea 21 research fellowship from the Korea Ministry of Education.

References

[1] V. Azaïs-Braesco, G. Pascal, Vitamin A in pregnancy: requirements and safety limits, Am. J. Clin. Nutr. 71 (Suppl.) (2000) 1325S–1333S.
[2] P. Bagavandoss, A.R. Midgley Jr., Biphasic action of retinoids on gonadotropin receptor induction in rat granulose cells in vitro, Life Sci. 43 (1988) 1607–1614.
[3] T.J. Barrett, T.C. Spelsberg, Steroid receptors at the nexus of transcriptional regulation, J. Cell. Biochem. Suppl. 30–31 (1998) 185–193.
[4] J.M. Bruder, A.J. Spaulding, M.E. Wierman, Phorbol ester inhibition of rat gonadotropin-releasing hormone promoter activity: role of Fos and Jun in the repression of transcription, Mol. Endocrinol. 10 (1996) 35–44.
[5] P. Chambon, A decade of molecular biology of retinoic acid receptors, FASEB J. 10 (1996) 940–954.
[6] U.R. Chandran, B.S. Warren, C.T. Baumann, G.L. Hager, D.B. DeFranco, The glucocorticoid receptor is tethered to DNA-bound Oct-1 at the mouse gonadotropin-releasing hormone distal negative glucocorticoid response element, J. Biol. Chem. 274 (1999) 2372–2378.
[7] B.P. Chew, Effects of supplemental beta-carotene and vitamin A on reproduction in swine, J. Anim. Sci. 71 (1993) 247–252.
[8] S. Cho, J. Han, W. Sun, D. Choi, H.B. Kwon, H. Jarry, W. Wuttke, K. Kim, Evidence for autocrine inhibition of gonadotropin-releasing hormone gene transcription by GnRH in hypothalamic GT1-1 neuronal cells, Mol. Brain Res. 50 (1997) 51–58.
[9] S. Cho, H. Cho, D. Geum, K. Kim, Retinoic acid regulates gonadotropin-releasing hormone release and gene expression in the rat hypothalamic fragments and GT1-1 neuronal cells in vitro, Mol. Brain Res. 54 (1998) 74–84.
[10] M.E. Clark, P.L. Mellon, The POU homeodomain transcription factor Oct-1 is essential for activity of the gonadotropin-releasing hormone neuron-specific enhancer, Mol. Cell. Biol. 15 (1995) 6169–6177.
[11] H. de Thé, M.M. Vivanco-Ruiz, P. Tiollais, H. Stunnenberg, A. Dejean, Identification of a retinoic acid responsive element in the retinoic acid receptor β gene, Nature 343 (1990) 177–180.
[12] J.D. Dignam, R.M. Lebovitz, R.G. Roeder, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, Nucleic Acids Res. 11 (1983) 1475–1489.
[13] S.A. Eraly, P.L. Mellon, Regulation of GnRH transcription by protein kinase C is mediated by evolutionarily conserved, promoter-proximal elements, Mol. Endocrinol. 9 (1995) 848–859.
[14] S.A. Eraly, S.B. Nelson, K.M. Huang, P.L. Mellon, Oct-1 binds

promoter elements required for transcription of the gonadotropin-releasing hormone gene, Mol. Endocrinol. 12 (1998) 469–481.
[15] S.R. Fox, R.E. Harlan, B.D. Shivers, D.W. Pfaff, Chemical characterization of neuroendocrine targets for progesterone in the female rat brain and pituitary, Neuroendocrinology 51 (1990) 276–283.
[16] I.C. Gaemers, A.M. van Pelt, P.T. van der Saag, J.W. Hoogerbrugge, A.P. Themmen, D.G. de Rooij, Effect of retinoid status on the messenger ribonucleic acid expression of nuclear retinoid receptors alpha, beta and gamma, and retinoid X receptors alpha, beta, and gamma in the mouse testis, Endocrinology 138 (1997) 1544–1551.
[17] V. Giguere, Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling, Endocr. Rev. 15 (1994) 61–79.
[18] C.K. Glass, Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers, Endocr. Rev. 15 (1994) 391–407.
[19] A.C. Gore, J.L. Roberts, Regulation of gonadotropin-releasing hormone gene expression in vivo and in vitro, Front. Neuroendocrinol. 18 (1997) 209–245.
[20] R. Heiermann, M. Rentrop, E. Lang, A. Mealicke, Cloning of several genes coding for retinoic acid receptors in the mouse embryonal carcinoma cell line PCC7-MZ1, J. Recept. Res. 13 (1993) 693–709.
[21] H.S.F. Huang, I. Dyrenfurth, G.L. Gunsalus, W.C. Hembree, Effect of vitamin A deficiency upon gonadotropin response to gonadotropin-releasing hormone, Biol. Reprod. 33 (1985) 1176–1187.
[22] P. Jansa, J. Forejt, A novel type of retinoic acid response element in the second intron of the mouse H2Kb gene is activated by the RAR/RXR heterodimer, Nucleic Acids Res. 24 (1996) 694–701.
[23] J.K. Kepa, B.M. Jacobsen, E.A. Boen, P. Prendergast, D.P. Edwards, G. Takimoto, M.E. Wierman, Direct binding of progesterone receptor to non-consensus DNA sequences represses rat GnRH, Mol. Cell. Endocrinol. 117 (1996) 27–39.
[24] J.K. Kepa, C.I. Neeley, B.M. Jacobsen, J.M. Bruder, D.P. McDonnell, K.K. Leslie, M.E. Wierman, Estrogen receptor mediated repression of rat gonadotropin-releasing hormone (GnRH) promoter activity in hypothalamic cells, Endocrine 2 (1994) 947–956.
[25] J.K. Kepa, C. Wang, C.I. Neeley, M.V. Raynolds, D.F. Gordon, W.M. Wood, M.E. Wierman, Structure of the rat gonadotropin-releasing hormone (rGnRH) gene promoter and functional analysis in hypothalamic cells, Nucleic Acids Res. 20 (1992) 1393–1399.
[26] K. Kim, J.Y. Seong, S.S. Kang, W. Sun, Neuroendocrine regulation of GnRH gene expression, in: I.S. Parhar, Y. Sakuma (Eds.), GnRH Neurons: Gene to Behavior, Brain Shuppan, Tokyo, 1997, pp. 401–427.
[27] Z.M. Lei, C.V. Rao, Cis-acting elements and trans-acting proteins in the transcriptional inhibition of gonadotropin-releasing hormone gene by human chorionic gonadotropin in immortalized hypothalamic GT1-7 neurons, J. Biol. Chem. 272 (1997) 14365–14371.
[28] B.F. Luisi, W.X. Xu, Z. Otwinowski, L.P. Fredman, K.R. Yamamoto, P.B. Sigler, Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA, Nature 352 (1991) 495–505.
[29] D.J. Mangelsdorf, R.M. Evans, The RXR heterodimers and orphan receptors, Cell 83 (1995) 841–850.
[30] D.J. Mangelsdorf, S.A. Kliewer, A. Kakizuka, K. Umesono, R.M. Evans, Retinoid receptors, Recent Prog. Horm. Res. 48 (1993) 99–121.
[31] E.M. McInerney, D.W. Rose, S.E. Flynn, S. Westin, T.-M. Mullen, A. Krones, J. Inostroza, J. Torchia, R.T. Nolte, N. Assa-Munt, M.V. Milburn, C.K. Glass, M.G. Rosenfeld, Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation, Genes Dev. 12 (1998) 3357–3368.
[32] C.A. Meier, Regulation of gene expression by nuclear hormone receptors, J. Recept. Signal Transduct. Res. 17 (1997) 319–335.
[33] S. Minucci, K. Ozato, Retinoid receptors in transcriptional regulation, Curr. Opin. Genet. Dev. 6 (1996) 567–574.
[34] S.B. Nelson, S.A. Eraly, P.L. Mellon, The GnRH promoter: target of transcription factors, hormones, and signaling pathways, Mol. Cell. Endocrinol. 140 (1998) 151–155.
[35] S.K. Nordeen, Luciferase reporter gene vectors for analysis of promoters and enhancers, Biotechniques 6 (1988) 454–457.
[36] K. Orchard, G. Lang, J. Harris, M. Collins, D. Latchman, A palindromic element in the human immunodeficiency virus long terminal repeat binds retinoic acid receptors and can confer retinoic acid responsiveness on a heterologous promoter, J. Acquir. Immune Defic. Syndr. 6 (1993) 440–445.
[37] B.D. Raisher, T. Gulick, Z. Zhang, A.W. Strauss, D.D. Moore, D.P. Kelly, Identification of a novel retinoid-responsive element in the promoter region of the medium chain acyl-coenzyme A dehydrogenase gene, J. Biol. Chem. 267 (1992) 20264–20269.
[38] R.C. Ribeiro, P.J. Kushner, J.D. Baxter, The nuclear hormone receptor gene superfamily, Annu. Rev. Med. 46 (1995) 443–453.
[39] W.J. Roesler, G.R. Vandenbark, R.W. Hanson, Cyclic AMP and the induction of eukaryotic gene transcription, J. Biol. Chem. 263 (1988) 9063–9066.
[40] S.A. Ross, P.J. McCaffery, U.C. Drager, L.M. De Luca, Retinoids in embryonal development, Physiol. Rev. 80 (2000) 1021–1054.
[41] A. Rowe, Retinoid X receptors, Int. J. Biochem. Cell Biol. 29 (1997) 275–278.
[42] J.W.R. Schwabe, L. Chapman, J.T. Finch, D. Rhodes, The crystal structure of the estrogen receptor DNA-binding domain bound to DNA: how receptors discriminate between their response elements, Cell 75 (1993) 567–578.
[43] R.D. Semba, Vitamin A, immunity and infection, Clin. Infect. Dis. 19 (1994) 489–499.
[44] J.Y. Seong, K. Kim, Measurement of GnRH mRNA level by Northern blot hybridization assay and competitive RT-PCR in the rat preoptic area, Neurosci. Protoc. 96–020–03 (1996) 1–17.
[45] B.D. Shivers, R.E. Harlan, J.I. Morell, D.W. Pfaff, Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurons, Nature 304 (1983) 345–347.
[46] S.M. Smith, E.D. Dickman, S.C. Power, J. Lancman, Retinoids and their receptors in vertebrate embryogenesis, J. Nutr. 128 (1998) 467S–470S.
[47] W. Sun, Y.S. Choe, Y.J. Lee, K. Kim, Suppression of GnRH gene expression in GT1-1 hypothalamic neuronal cells: action of protein kinase C, Neuroreport 8 (1997) 3541–3546.
[48] K. Umesono, K.K. Murakami, C.C. Thompson, R.M. Evans, Direct repeats as selective response elements for the thyroid hormone, retinoic acid and vitamin D3 receptors, Cell 65 (1991) 1255–1266.
[49] N.L. Weigel, Y. Zhang, Ligand-independent activation of steroid hormone receptors, J. Mol. Med. 76 (1998) 469–479.
[50] S.L. Whaley, V.S. Hedgpeth, C.E. Farin, N.S. Martus, F.C.L. Jayes, J.H. Britt, Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets, J. Anim. Sci. 78 (2000) 1598–1607.
[51] M.E. Wierman, X. Xiong, J.K. Kepa, A.J. Spaulding, B.M. Jacobsen, Z. Fang, G. Nilaver, S.R. Ojeda, Repression of gonadotropin-releasing hormone promoter activity by the POU homeodomain transcription factor SCIP/Oct-6/Tst-1: a regulatory mechanism of phenotype expression?, Mol. Cell. Biol. 17 (1997) 1652–1685.
[52] S.B. Wolbach, P.R. Howe, Tissue changes following deprivation of fat-soluble vitamin A, J. Exp. Med. 42 (1925) 753–760.
[53] M. Zakaria, I.C. Dunn, S. Zhen, E. Su, E. Smith, E. Patriquin, S. Radovick, Phorbol ester regulation of the gonadotropin-releasing hormone (GnRH) gene in GnRH-secreting cell lines: a molecular basis for species difference, Mol. Endocrinol. 10 (1996) 1282–1291.
[54] A. Zelent, A. Krust, M. Petkovich, P. Kastner, P. Chambon, Cloning of murine α and β retinoic acid receptors and a novel receptor γ predominantly expressed in skin, Nature 339 (1989) 714–717.