ELSEVIER

Molecular Brain Research 87 (2001) 214–222

MOLECULAR BRAIN RESEARCH

www.elsevier.com/locate/bres

Research report

9-*cis*-Retinoic acid represses transcription of the gonadotropin-releasing hormone (GnRH) gene via proximal promoter region that is distinct from all-*trans*-retinoic acid response element

Sehyung Cho[a], Jean-Ju Chung[a], Jin Han[a], Byung Ju Lee[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]*Department of Biology, University of Ulsan, Ulsan 680-749, South Korea*
[c]*Department of Life Science, Kwangju Institute of Science and Technology, Kwangju 500-712, South Korea*

Accepted 2 January 2001

Abstract

We previously reported an enhancing effect of all-*trans*-retinoic acid (all-*trans*-RA) on gonadotropin-releasing hormone (GnRH) gene transcription via distal promoter elements of the rat GnRH gene. The present study examined the effects of another biologically active retinoid, 9-*cis*-retinoic acid (9-*cis*-RA), on GnRH transcription in GT1-1 cells. Similar to the action of all-*trans*-RA, 9-*cis*-RA significantly induced the luciferase activity of the strong retinoic acid response element (RARE) reporter construct, 3XβRARE-Luc, by about 60-fold, indicating that GT1-1 cells are also responsive to 9-*cis*-RA. In contrast to the stimulatory effect of all-*trans*-RA on GnRH transcription, 9-*cis*-RA inhibited the GnRH promoter activity in a dose- and time-dependent manner. Significant inhibition by 9-*cis*-RA required at least an 18 h treatment and a further decrease of GnRH promoter-driven luciferase activity was observed up to 48 h of incubation. Accordingly, GnRH mRNA levels were decreased by 9-*cis*-RA treatment in a similar dose- and time-related manner, indicating that mouse GnRH expression is also negatively regulated by 9-*cis*-RA. Transient transfections of serial deletion constructs of the rat GnRH promoter revealed that the −230/−110 sequence of the rat GnRH promoter is responsible for 9-*cis*-RA-induced inhibition of GnRH transcription. Within this region, however, no consensus retinoid X receptor response element was found. To gain insights into the role of retinoid X receptors (RXRs) in GnRH expression, we examined the effects of RXR overexpression on GnRH transcriptional activity. Interestingly, co-transfection of RXR overexpression vectors significantly increased the GnRH promoter-driven luciferase activity, while treatment with 9-*cis*-RA not only nullified the enhancing effect of RXR overexpression but also decreased the basal GnRH promoter-driven luciferase activity by 50% compared to vehicle-treated controls. This implies that RXRs in the absence of its cognate ligand 9-*cis*-RA contribute to the maintenance of basal GnRH gene transcription. Northern blot analysis revealed that 9-*cis*-RA, but not all-*trans*-RA, down-regulated RXRβ expression in GT1-1 cells, suggesting that one possible mechanism of 9-*cis*-RA-induced repression involves down-regulation of RXR expression. In conclusion, the present study clearly demonstrates that 9-*cis*-RA is a negative regulator of GnRH gene expression in immortalized GnRH neurons.

Theme: Endocrine and autonomic regulation

Topic: Hypothalamic–pituitary–gonadal regulation

Keywords: Gonadotropin-releasing hormone, 9-*cis*-Retinoic acid, Retinoid X receptor; GT1-1 cell; Transcriptional regulation

1. Introduction

Retinoids (vitamin A derivatives) play important roles in diverse biological processes including development, differentiation, vision, immunitiy, and reproduction (reviewed in [32,42,43]). Nearly all functions of vitamin A except vision are accomplished by two active metabolites of vitamin A, all-*trans*-retinoic acid (all-*trans*-RA) and 9-*cis* retinoic acid (9-*cis* RA) which bind to and activate specific nuclear hormone receptors (reviewed in [6,15,27,29,37]). There exist two subfamilies of retinoid receptors, retinoic

*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)00020-1

acid receptors (RARs) and retinoid X receptors (RXRs), which belong to the superfamily of steroid/thyroid/vitamin D/retinoid hormone receptors. Each subfamily of the retinoid receptors consists of three distinct receptors, α, β, and γ, encoded by separate genes on six different chromosomes. All-*trans*-RA activates only RARs, whereas 9-*cis*-RA is known to activate both RARs and RXRs. 9-*cis*-RA is believed to activate only RXRs in the presence of cellular retinoid binding proteins under normal physiological conditions [32,35]. In addition to the role as 9-*cis*-RA receptors (reviewed in [44]), RXRs also function as common heterodimeric partners for other nuclear hormone receptors such as RARs, vitamin D_3 receptors (VD_3Rs), triiodothyronine receptors (T_3Rs), and peroxisome proliferator-activated receptors (PPARs) (reviewed in [16,26,37]). Thus, RXR is a critical component of heterodimeric formation, which in turn generates diverse hormonal responses [26].

Although it has long been known that retinoids are essential for normal reproductive functions, most studies had focused primarily on male germ cell development (reviewed in [8,17,40]) and no studies were available concerning the effect of retinoids on gonadotropin-releasing hormone (GnRH) neurons. In the previous study, we provided for the first time evidence that all-*trans*-RA may be an important regulator of GnRH neurons [9]. We demonstrated that the GnRH neuronal cell line (GT1-1) as well as the hypothalamic tissues possess functional retinoid receptors including RARα, RARγ, RXRα and RXRβ, and that all-*trans*-RA stimulates GnRH secretion and increases GnRH mRNA levels via enhanced gene transcription. In the accompanying paper, we localized the functional retinoic acid response element (RARE) at −1494/−1470 of the rat GnRH promoter.

In the present study, we investigated the effect of 9-*cis*-RA on the GnRH gene transcription. In contrast to the stimulatory effect of all-*trans*-RA, 9-*cis*-RA reduced the GnRH mRNA levels via transcriptional repression. Moreover, 9-*cis*-RA-induced repression was mediated by the proximal promoter elements that are distinct from the all-*trans*-RA responsive element.

2. Materials and methods

2.1. Materials

9-*Cis*-RA was obtained from BIOMOL (Plymouth Meeting, PA, USA). Other hormones including the all-*trans*-RA, 1α,25-dihydroxycholecalciferol and triiodothyronine were from Sigma (St. Louis, MO, USA). All materials for cell culture were purchased from Gibco-BRL (Grand Island, NY, USA). Other chemicals, if not mentioned otherwise, were from Sigma. The plasmids pGnRH3.0-LUC and 3XβRARE-LUC were described previously [9]. The murine RXR expression vectors were generous gifts from Dr. P. Chambon (Institut de Chimie Biologique, Strasbourg Cédex, France). pCMV-βgal which contains the cytomegaloviral promoter fused to the β-galactosidase gene was obtained from Stratagene (La Jolla, CA, USA) and were used to compensate transfection efficiency. Serial deletion constructs of the rat GnRH promoter were made by ExoIII digestion using the Erase-A-Base® system (Promega, Madison, WI, USA) or by PCR-based cloning strategy. The sequences of the constructs were confirmed with the dideoxy sequencing method.

2.2. GT1-1 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. Two days before retinoid treatment, 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.

2.3. Transient transfection

GT1-1 cells were plated in 60-mm tissue culture 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. Two 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 GmbH, Hilden, Germany) according to the 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 resulting 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. For co-transfection of RXR overexpression vectors, the amount of β-galactosidase reporter vector was reduced and RXR overexpression vectors or carrier DNAs were added to maintain the constant amount of DNA per dish. 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 Co., 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 Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard. β-Galactosidase and luciferase assays were 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.

2.4. Northern blot hybridization analysis

Detailed procedure for Northern blot hybridization was described elsewhere [10]. Briefly, total RNA from GT1-1 cells was extracted by acid guanidinium thiocyanate–phenol–chloroform method [11]. Ten μg of total RNA was denatured, resolved in an 1.2% formaldehyde gel, and transferred to Nylon membranes (Schleicher & Schuell). The membranes were prehybridized at 42°C for 2 h and then hybridized for 16–24 h with ^{32}P-labeled specific probes as described below. After autoradiography, membranes were rehybridized with 18S cDNA probes. Relative mRNA levels were normalized with 18S RNA signals by quantitative densitometric scanning of the autoradiograms (Hoeffer Scientific Instruments). The probe used to detect the mouse GnRH mRNA in GT1-1 cells was a 396 bp complementary DNA (cDNA) fragment which is identical to 8–403 fragment of the mouse GnRH cDNA coding sequence [28]. The probe used to detect the mouse RXRβ mRNA was described previously [9]. All probes were random primer-labeled with α-[^{32}P]-dCTP.

2.5. Statistical analysis

Data for GnRH promoter activity and mRNA level 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. 9-cis-RA represses the rat GnRH promoter activity in a dose- and time-dependent manner

We previously demonstrated that all-*trans*-RA increased rat GnRH promoter activity as well as GnRH mRNA levels in the GT1-1 cells in a dose- and time-related manner [9]. Since we observed that GT1-1 cells express both RARs and RXRs, we hypothesized that GnRH neurons might be also responsive to another biologically active retinoid, 9-*cis*-RA. To test this possibility, we transfected the GT1-1 cells with the strong retinoic acid response element (RARE) reporter construct 3XβRARE-LUC and compared the effects of various lipophilic hormones including 9-*cis*-RA on the induction of luciferase activity (Fig. 1A). As reported previously [9], all-*trans*-RA induced the luciferase activity by about 80-fold.

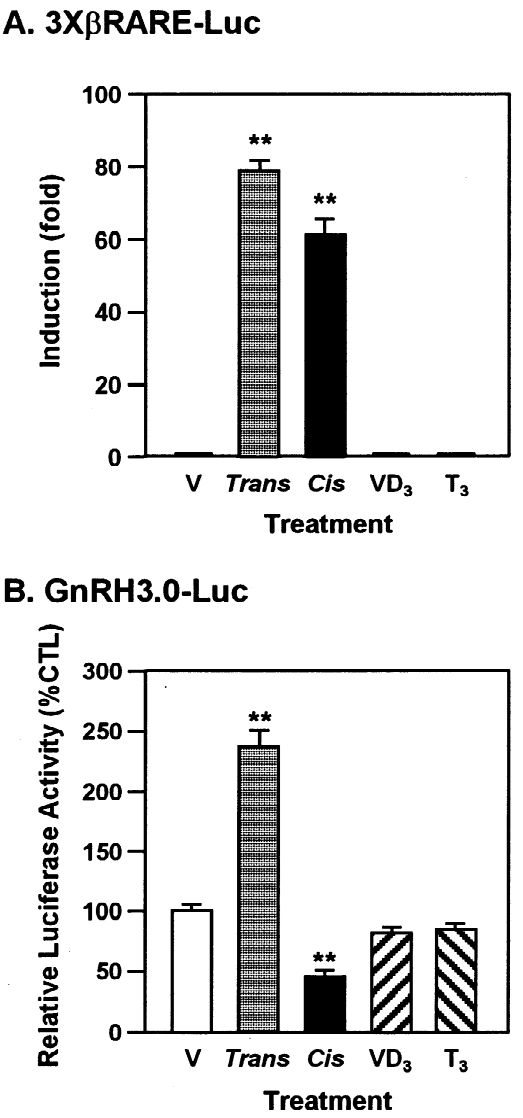

Fig. 1. 9-*cis*-RA decreases the rat GnRH promoter activity, while all-*trans*-RA increases it. (A) Effects of various nuclear receptor ligands on the induction of strong RARE reporter activity in GT1-1 cells. GT1-1 cells were transfected with 3XβRARE-LUC and treated with vehicle or 1 μM of ligands as indicated. One day after treatment, cell extracts were prepared and analyzed for luciferase activity ($n=6$). (B) Effects of various nuclear receptor ligands on the rat GnRH promoter activity in GT1-1 cells. Cells were transfected with pGnRH3.0LUC and treated with vehicle or 1 μM of ligands as indicated. Luciferase activity was measured 1 day after treatment ($n=6$). V: vehicle, *Trans*: all-*trans*-RA, *Cis*: 9-*cis*-RA, VD_3: 1α,25-dihydroxycholecalciferol, T_3: triiodothyronine.

Treatment of GT1-1 cells with 9-*cis*-RA also induced the luciferase activity by about 60-fold, whereas other lipophilic hormones such as vitamin D_3 (VD_3) or triiodothyronine (T_3) had no effect. These results imply that GT1-1 cells are responsive to 9-*cis*-RA. Then we tested the effect of 9-*cis*-RA on transcription of the rat GnRH gene. GT1-1 cells were transfected with a GnRH promoter-driven luciferase reporter construct (pGnRH3.0-LUC) and treated with various lipophilic hormones (1 μM each) for 24 h. As described previously [9], all-*trans*-RA induced the luciferase activity to around 250% of the vehicle-treated control group. In contrast, 9-*cis*-RA significantly ($P<0.01$) reduced the luciferase activity to about 50% of the control value (Fig. 1B). This repression of the GnRH promoter activity by 9-*cis*-RA appears specific, since other nuclear hormones (VD_3 or T_3) had little effect on the GnRH promoter-driven luciferase activity.

To confirm the repressive effect of 9-*cis*-RA on the GnRH transcription, dose–response and time-course changes of GnRH promoter activity were examined. Treatment of GT1-1 cells with 9-*cis*-RA for 24 h decreased the rat GnRH promoter activity in a dose-related manner. As shown in Fig. 2A, a low concentration of 9-*cis*-RA (1 nM) significantly decreased the rat GnRH promoter activity by 25%. Maximal inhibition (50% decrease from the vehicle-treated control) was seen in GT1-1 cells treated with 100 nM or higher concentrations of 9-*cis*-RA. Time-course experiments revealed that 9-*cis*-RA (1 μM)-induced repression of the GnRH promoter activity required at least 18 h incubation with 9-*cis*-RA (Fig. 2B). In addition, treatment of GT1-1 cells with 9-*cis*-RA for 48 h further decreased the luciferase activity to 30–35% of the control level.

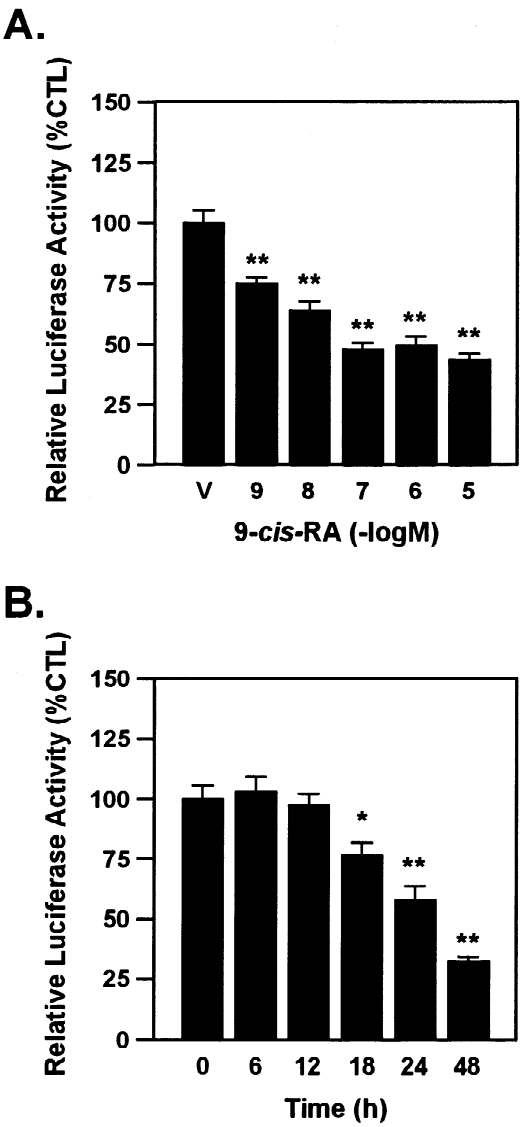

Fig. 2. 9-*cis*-RA decreases the rat GnRH promoter activity in GT1-1 cells in a dose- and time-related manner. (A) Dose-response effects of 9-*cis*-RA on the rat GnRH promoter activity. GT1-1 cells transfected with pGnRH3.0LUC were treated with 10^{-9}–10^{-5} M 9-*cis*-RA for 24 h and then analyzed for luciferase activity ($n=6$). (B) Time-course effects of 9-*cis*-RA on the rat GnRH promoter activity. GT1-1 cells transfected with pGnRH3.0LUC were treated with 1 μM 9-*cis*-RA for 0–48 h. Data are expressed as percentage of the control value ($n=6$). *$P<0.05$ vs. control; **$P<0.01$ vs. control.

3.2. 9-cis-RA also decreases the GnRH mRNA level in a dose- and time-dependent manner

We then examined whether the transcriptional activity of the endogenous GnRH gene was reduced by 9-*cis*-RA treatment in GT1-1 mouse cells (Fig. 3). The results revealed that 9-*cis*-RA reduced GnRH mRNA levels in a dose- and time-dependent manner. As shown in Fig. 3A, about 40–50% decreases were observed in GT1-1 cells treated with 9-*cis*-RA for 24 h at or higher than 100 nM. In addition, incubations with 9-*cis*-RA (1 μM) longer than 12 h were required for a significant decrease compared to vehicle-treated control (Fig. 3B). GnRH mRNA levels in cells treated with 9-*cis*-RA remained low up to 48 h incubation. Thus, both the transfected rat GnRH promoter activity and the endogenous mouse GnRH mRNA levels were down-regulated by 9-*cis*-RA in a similar dose- and time-related manner, suggesting that 9-*cis*-RA represses GnRH expression at the level of transcription.

3.3. Transcriptional repression by 9-cis-RA is mediated by a proximal region of the rat GnRH promoter

To localize the promoter region responsible for the 9-*cis*-RA action, we prepared serial deletion constructs of the rat GnRH promoter linked to a reporter gene. We first examined the relative basal luciferase activity of each deletion construct (Table 1). The largest construct −3002GnRH-LUC exhibited a high luciferase activity of 3×10^5 to 1×10^6 relative light unit (RLU) per 20 μl of

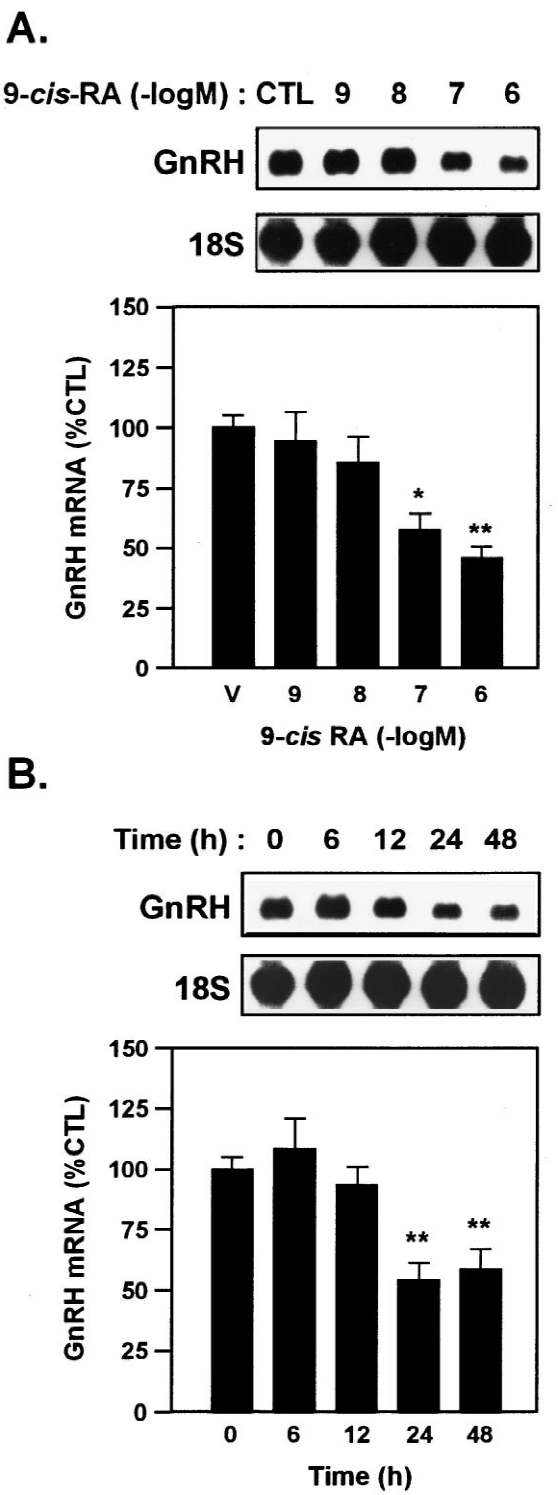

Fig. 3. Long-term effects of 9-*cis*-RA on GnRH mRNA levels in GT1-1 cells. (A) Dose-response effects of 9-*cis*-RA on GnRH mRNA levels in GT1-1 cells incubated with 10^{-9}–10^{-6} M 9-*cis*-RA for 24 h. A representative autoradiogram of Northern blot analysis (upper panel) and results obtained from three independent experiments (lower panel) are shown. (B) Time-course effect of 9-*cis*-RA on GnRH mRNA levels. GT1-1 cells were incubated with 9-*cis*-RA (1 μM) for 0–48 h and GnRH mRNA levels were determined by Northern blot analysis. A representative autoradiogram (upper panel) and results obtained from three independent experiments (lower panel) are shown. *$P<0.05$ vs. control; **$P<0.01$ vs. control.

Table 1
Basal promoter activity of the serial deletion constructs of the rat GnRH promoter as measured by luciferase activity in GT1-1 cells[a]

GnRH promoter construct	Relative luciferase activity (% of largest construct)
−3002GnRH-LUC	100±6.2
−2264GnRH-LUC	153±5.8
−1640GnRH-LUC	35.1±3.1
−1438GnRH-LUC	30.8±4.6
−854GnRH-LUC	22.5±3.4
−230GnRH-LUC	5.2±0.38
−110GnRH-LUC	1.7±0.09
−81GnRH-LUC	0.1±0.01
Promoterless	<0.02

[a] Serial deletion constructs of the rat GnRH promoter fused to luciferase reporter vector were introduced into GT1-1 cells by a modified calcium phosphate co-precipitation procedure. Data are expressed as mean±S.E.M. of the percentage of the largest construct ($n=6$).

extracts. Deletion from −3002 to −2264 reproducibly increased the luciferase activity by about 50%, suggesting the presence of an inhibitory element(s) within this region. In contrast, deletion to −1640 decreased the luciferase activity to 35% compared to the largest construct. This is in concurrence with previous reports that neuron-specific enhancer (NSE) elements are localized in −1863/−1571 of the rat GnRH promoter [46]. Further deletion up to −854 had marginal effect on the basal promoter activity, but deletion to −230, −110 or to −81 gradually decreased the promoter activity to 5.2%, 1.7% and 0.1% levels of the largest construct, respectively. These results are consistent with previous reports [9,22].

The response of each deletion construct to 9-*cis*-RA were subsequently examined following transient transfections. As expected, the largest construct −3002GnRH-LUC responded to 9-*cis*-RA by decreasing luciferase activity by around 50% (Fig. 4). Further deletions up to −230 still showed qualitatively the same responses to 9-*cis*-RA treatment. Of note is that the deletion from −1640 to −1438, which was previously shown to confer responsiveness to all-*trans*-RA [9], had no effect on the responsiveness to 9-*cis*-RA. However, deletion to −110 completely abolished the responsiveness of the reporter construct to 9-*cis*-RA treatment. The promoterless vector showed low but measurable luciferase activity (<0.02% of the largest construct) and did not respond to 9-*cis*-RA at all. These results clearly indicate that a proximal element within −230/−110 of the rat GnRH gene is responsible for 9-*cis*-RA-induced transcriptional repression.

3.4. Co-transfection of RXRα increases GnRH promoter activity in the absence of its cognate ligand 9-cis-RA

We previously demonstrated that GT1-1 cells contain high levels of RXRβ and low levels of RXRα transcripts [9]. To examine the role of RXRs in the maintenance of

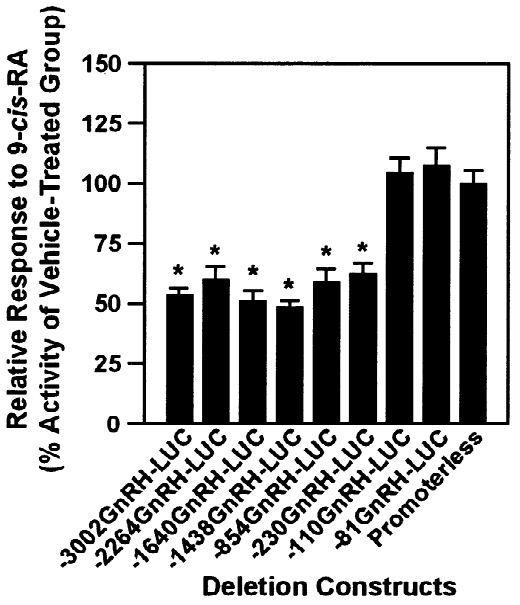

Fig. 4. Effects of 9-*cis*-RA on 5′-deletional constructs of the rat GnRH promoter. Serial deletion constructs were made by Exo III digestion or by PCR-based cloning strategy. End-points were determined by conventional dideoxy sequencing. Cells were transfected with each construct and treated with vehicle or 1 μM 9-*cis*-RA for 24 h. Data are expressed as mean percentage±S.E.M. of the vehicle treated control of each deletion construct ($n=6$). *$P<0.01$ vs. control.

GnRH promoter activity, mammalian overexpression vectors for RXRα and RXRβ were co-transfected with the GnRH promoter reporter construct (pGnRH3.0Luc). As shown in Fig. 5, overexpression of RXRα increased the GnRH promoter activity to about 170% of the control,

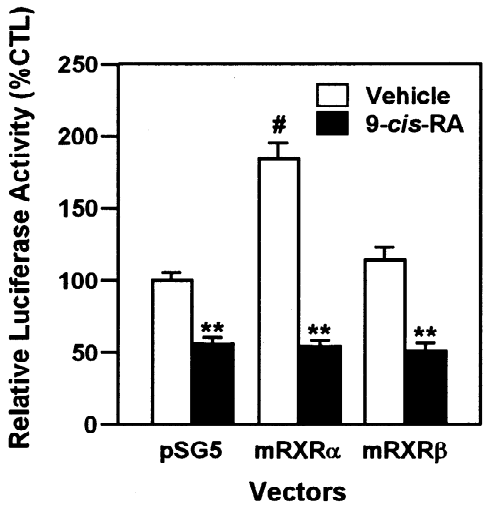

Fig. 5. Cotransfection of murine RXRα expression vectors increases the rat GnRH promoter activity when unstimulated with 9-*cis*-RA. GT1-1 cells were cotransfected with pGnRH3.0LUC (5 μg/dish) and either with murine RXRα or RXRβ expression vectors (2 μg/dish). DNA amounts were adjusted to 15 μg/dish with carrier DNA. Cells were treated with vehicle or 9-*cis*-RA (1 μM) for 24 h and analyzed for luciferase activity. # $P<0.01$ vs. vehicle-treated control which was not cotransfected with RXR expression vector. **$P<0.01$ vs. vehicle-treated each control.

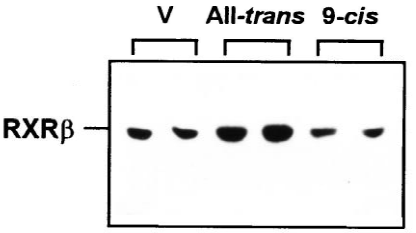

Fig. 6. Northern blot analysis of RXRβ gene expression in GT1-1 cells following retinoid treatment. Total RNA was isolated from GT1-1 cells treated with vehicle, all-*trans*-RA (1 μM) or 9-*cis*-RA (1 μM) for 24 h. Thirty micrograms of RNA was loaded on each lane and resolved on a 1% formaldehyde gel. After transferring to Nytran membrane, membranes were hybridized with ^{32}P-labeled RXRβ-specific cDNA probes. Northern blot analysis was repeated at least three times with different RNA preparations. Representative autoradiogram is shown here.

whereas that of RXRβ showed little effect. However, treatment of RXRα-overexpressing cells with 9-*cis*-RA decreased the GnRH promoter activity to the 9-*cis*-RA-treated control level. This indicates that RXRα increases GnRH promoter activity in the absence of its endogenous ligand 9-*cis*-RA.

3.5. Opposite effects of 9-cis-RA and all-trans-RA on RXRβ mRNA level

To gain insight into the function of RXRs, changes in mRNA levels encoding RXRs were analyzed following retinoid treatment. GT1-1 cells were treated with either all-*trans*-RA (1 μM) or 9-*cis*-RA (1 μM) for 24 h and total RNA was isolated. RXR gene expression was analyzed by Northern blot hybridization with subtype-specific probes as described previously [9]. RXRγ mRNA was below the detection limit in GT1-1 cells. Only marginal expression of RXRα was observed in GT1-1 cells and was not affected by retinoid treatment (data not shown). However, 9-*cis*-RA significantly reduced the mRNA level of RXRβ, while all-*trans*-RA significantly induced it (Fig. 6). Combined with the results obtained by co-transfection experiments, the present data suggest one possible mechanism by which 9-*cis*-RA-induced inhibition of GnRH gene transcription can be mediated by repression of RXRβ gene expression. It should be determined in the future experiments whether changes in RXRβ mRNA levels reflect alterations in RXRβ protein levels.

4. Discussion

The present study clearly demonstrated that 9-*cis*-RA down-regulates GnRH gene expression presumably via an indirect transcriptional repression mechanism. In contrast to the stimulatory effect of all-*trans*-RA on GnRH gene transcription [9], the inhibitory effect of 9-*cis*-RA was mediated by proximal promoter element(s) located between −230 and −110 from the major transcription start site,

where no RXRE or related sequence element exists. Thus, the transcriptional repression by 9-*cis*-RA seems to be indirectly mediated by other transcription factors binding to proximal elements of the GnRH promoter, while transcriptional activation by all-*trans*-RA is directly mediated by its cognate receptors binding to the distal RARE [see accompanying paper]. Of note is that 9-*cis*-RA also decreased GnRH mRNA levels in a similar dose- and time-related manner in the mouse-derived GT1-1 cells (Fig. 3), along with a decrease of the transfected rat GnRH promoter activity (Fig. 2). This raises a possibility that the 9-*cis*-RA similarly represses the mouse GnRH gene transcription. Indeed, the proximal promoter regions of the rat, mouse and human GnRH are relatively well conserved ([33] and Refs. therein).

The opposite regulation of GnRH gene expression by all-*trans*-RA and 9-*cis*-RA at the transcription level is an interesting finding. Differential actions of 9-*cis*-RA from its stereoisomer all-*trans*-RA have been reported by other investigators. For example, it was reported that differentiation and proliferation of neuroblastoma cells in vitro was regulated in different manners by two active retinoids [34] and possibly involves retinoid isomer-specific induction of nuclear receptor coregulators [24]. In another study, 9-*cis*-RA was found to diminish the all-*trans*-RA-induced spermatogenesis in vitamin A-deficient mice, although 9-*cis*-RA alone was able to induce spermatogenesis in this animal [14]. Moreover, Carlberg et al. [5] provided evidence that 9-*cis*-RA can be a natural antagonist for the retinoic acid response pathway. Although all-*trans*-RA is known to interact only with RARs and 9-*cis*-RA with both RARs and RXRs in vitro ([1,2] and Refs. therein), the presence of retinoid binding proteins seems to restrict 9-*cis*-RA to bind only to RXRs in vivo [32,35]. Since 9-*cis*-RA activation is known to dissociate RXRs from its heterodimeric partners and favors the formation of RXR–RXR homodimers, the amount of RXRs available for the formation of RAR–RXR heterodimers will be greatly diminished by 9-*cis*-RA treatment. This squelching effect may in turn explain the opposite effect of all-*trans*-RA and 9-*cis*-RA on the transcription of target genes, as reported in another system [23]. In support of this notion, treatment of GT1-1 cells with vitamin D_3 or T_3, which would enhance the formation of VD_3R–RXR and T_3R–RXR heterodimer respectively, slightly but significantly ($P<0.05$) reduced the transfected rat GnRH promoter activity (about 10–15% decrease from the vehicle-treated control; see Fig. 1B). This notion is further supported by the finding that overexpression of RXRα increases the basal GnRH gene transcription, while activation of RXRs with 9-*cis*-RA nullifies this enhancing effect (Fig. 5). However, this enhancing effect of basal GnRH gene transcription was not observed with RXRβ. The reason for this is unclear, but the fact that GT1-1 cells express high levels of RXRβ and low levels of RXRα may explain this discrepancy [9].

Although the functions of RXRs as a common heterodimeric partner for various nuclear hormone receptors have been intensively studied, the functions of ligand-occupied RXRs (hence the function of RXR homodimers) or the functions of 9-*cis*-RA recently have been emerging. For example, ligand-bound RXRs seem to mediate the retinoid signal transduction during embryogenesis [25]. Moreover, examples of interactions between RXRs and other transcription factors are greatly expanding [12,38]. Of importance to the 9-*cis*-RA-induced repression of GnRH are the interactions of RXRs with steroid hormone receptors such as estrogen receptors or progesterone receptors as exemplified in the repression of the estrogen-induced expression of the very low density apolipoprotein II gene [41]. Interestingly, estrogen- and progesterone-induced repressions of the GnRH transcription were mapped to −171/−126 and −171/−73 of the rat GnRH promoter [20,21], which overlap with the promoter region responsible for 9-*cis*-RA-induced repression of the rat GnRH promoter. Thus, it is tempting to postulate the possible interactions between 9-*cis*-RA and steroid signaling cascade within this region. Kepa et al. [20] were able to show a direct binding of progesterone receptor to the DNA fragments −171/−126, −126/−73, −111/−73 by electrophoretic mobility shift assays in vitro using purified progesterone receptors. However, it should be noted that the presence of progesterone receptor is still unclear in GT1-1 cells as well as in hypothalamic tissues. Thus, other transcription factors yet unproved to bind the GnRH promoter should be considered.

A computer-aided search of putative transcription factor binding sites within −230/−110 of the rat GnRH promoter revealed two putative transcription factor binding sites, an incomplete heat shock factor binding site at −190 (5′-CTT**GAA**CT**TTC**CTC) and a relatively well conserved myeloid zinc finger-1 (MZF-1) binding site at −180 (5′-TC**AGCGGGGA**AT). Heat shock factors are known to bind as tetramers to DNA sequence elements of alternating palindromic repeats of 5′-n**GAA**n sequence (i.e., n**GAA**nn**TTC**nn**GAA**nn**TTC**n) [39]. Since the putative heat shock factor binding site in the rat GnRH promoter has only two 5′-n**GAA**n motifs, this element seems unlikely to be functional. In contrast, the putative MZF-1 binding site of the rat GnRH promoter is likely to be functional, since the putative MZF-1 binding site has only one mismatch at position 3 compared to the consensus MZF-1 binding site (5′-TC**AGTGGGGA**AT) [30] and we observed in a preliminary experiment that GT1-1 cells possess considerable amounts of proteins binding to the consensus MZF-1 binding site in the electrophoretic mobility shift assays. The binding activity to this site was reduced in a time-dependent manner upon 9-*cis*-RA treatment (data not shown). MZF-1 is a retinoic acid-responsive, myeloid-specific C_2H_2 zinc finger transcription factor whose cDNA was cloned originally from peripheral leukocytes [4,18]. Although the genomic DNA sequences

of human MZF-1 and transcriptional regulation of CD34 promoter by MZF-1 are available [19,31], the fine molecular mechanism of its action and expression of MZF-1 in nonmyeloid cells is largely unknown. Thus, it would be interesting to examine whether GnRH neurons in vivo and GT1-1 neuronal cells in vitro actually express the MZF-1 protein and how they regulate GnRH gene transcription in response to retinoids. It has been recently found that the highly conserved proximal promoter region of the rat GnRH gene is bound by multiple nuclear proteins containing POU-homeodomain transcription factor Oct-1 [13]. In addtion, the distal negative glucocorticoid response element (nGRE) located between −237 and −201 of the mouse GnRH gene is recognized by Oct-1 and glucocorticoid receptor interact directly with Oct-1 bound to this distal nGRE [7]. Thus the interaction of RXR with other transcription factors bound to proximal promoter region would be interesting.

Changes in RXR subtype gene expression comprise yet another possible mechanism of GnRH gene regulation by 9-*cis*-RA. We observed that overexpression of RXRα induced the basal GnRH gene transcription in the absence of its cognate ligand 9-*cis*-RA (Fig. 5). Although its relevance to the ligand-independent activation of nuclear receptor ([45] and Refs. therein) should be determined by future experiments, this supports a hypothesis that the intracellular concentration of RXRs that are available as heterodimeric partners to other nuclear receptors such as RARs are important for the maintenance of GnRH gene transcription. This scenario explains the observation that, when exposed to 9-*cis*-RA, the enhancing effect of RXR overexpression disappears. Moreover, 9-*cis*-RA repressed the basal GnRH transcription to around 50% of the control. In this case, the decrease in the basal GnRH gene transcription can be attributed in part to the RXR homodimer formation and in part to the down-regulation of RXR gene expression (Fig. 6). Thus, it seems clear that down-regulation of RXR expression may contribute, at least in part, to the 9-*cis*-RA-induced repression of the GnRH gene transcription.

The opposite regulation of GnRH gene expression by the two active metabolites of vitamin A adds more complexity to the physiological regulation of GnRH neuronal activity. It is possible that, by changing the relative concentration of each metabolite, retinoids may be involved in the fine-tuning of GnRH neuronal activity. Although the serum concentration of retinol is maintained within narrow range around 1 μM and that of RA around receptor K_d values, intracellular concentration of retinoids can be varied significantly depending on the presence and/or amount of cellular retinoid binding proteins and retinoid-synthesizing/converting/degrading enzymes [3,36,40]. In this respect, it would be interesting to examine whether GnRH neurons express these proteins and how these proteins are regulated.

In conclusion, the present study clearly demonstrated that 9-*cis*-RA down-regulates GnRH gene expression at the level of transcription via proximal promoter sequence that contains no consensus RXRE. The precise mechanism by which 9-*cis*-RA represses GnRH gene transcription awaits future experiments.

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 J. Han are supported by the Brain Korea 21 research fellowship from the Korea Ministry of Education.

References

[1] G. Allenby, M-T. Bocquel, M. Saunders, S. Kazmer, J. Speck, M. Rosenberger, A. Lovey, P. Kastner, J.F. Grippo, P. Chambon, A.A. Levin, Retinoic acid receptors and retinoid X receptors: Interaction with endogenous retinoic acids, Proc. Natl. Acad. Sci. USA 90 (1993) 30–34.

[2] G. Allenby, R. Janocha, S. Kazmer, J. Speck, J.F. Grippo, A.A. Levin, Binding of 9-cis-retinoic acid and all-trans retinoic acid to retinoic acid receptors α, β, and γ, J. Biol. Chem. 269 (1994) 16689–16695.

[3] R. Blomhoff, M.H. Green, J.B. Green, T. Berg, K.R. Norum, Vitamin A metabolism: new perspectives on absorption, transport, and storage, Phyiol. Rev. 71 (1991) 951–990.

[4] L. Bavisotto, K. Kaushansky, N. Lin, R. Hromas, Antisense oligonucleotides from the stage-specific myeloid zinc finger gene MZF-1 inhibit granulopoiesis in vitro, J. Exp. Med. 174 (1991) 1097–1101.

[5] C. Carlberg, Saurat, J-H. and Siegenthaler, G., 9-cis retinoic acid is a natural antagonist for the retinoic acid receptor response pathway, Biochem. J. 295 (1993) 343–346.

[6] P. Chambon, The molecular and genetic dissection of the retinoid signaling pathway, Recent Prog. Horm. Res. 50 (1995) 317–332.

[7] 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.

[8] B.P. Chew, Effects of supplemental beta-carotene and vitamin A on reproduction in swine, J. Anim. Sci. 71 (1993) 247–252.

[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] 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.

[11] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.

[12] M. Crestani, A. Sadeghpour, D. Stroup, G. Galli, J.Y. Chiang, The opposing effects of retinoic acid and phorbol esters converge to a

common response element in the promoter of the rat cholesterol 7 alpha-hydroxylase gene (CYP7A), Biochem. Biophys. Res. Commun. 225 (1996) 585–592.
[13] S.A. Eraly, S.B. Nelson, K.M. Huang, P.L. Mellon, Oct-1 binds promoter elements required for transcription of the GnRH gene, Mol. Endocrinol. 12 (1998) 469–481.
[14] I.C. Gaemers, E. Sonneveld, A.M. van Pelt, B.H. Schrans, A.P. Themmen, P.T. van der Saag, D.G. de Rooij, The effect of 9-cis-retinoic acid on proliferation and differentiation of a spermatogonia and retinoid receptor gene expression in the vitamin A-deficient mouse testis, Endocrinology 139 (1998) 4269–4276.
[15] V. Giguere, Retinoic acid receptors and cellular retinoid binding proteins: Complex interplay in retinoid signaling, Endocr. Rev. 15 (1994) 61–79.
[16] C.K. Glass, Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers, Endocr. Rev. 15 (1994) 391–407.
[17] M.D. Griswold, P.D. Bishop, K.H. Kim, R. Ping, J.E. Siiteri, C. Morales, Function of vitamin A in normal and synchronized seminiferous tubules, Ann. NY Acad. Sci. 564 (1989) 154–172.
[18] R. Hromas, S. Collins, D. Hickstein, W. Raskind, L. Deaven, P. O'Hara, F. Hagen, K. Kaushansky, A retinoic acid-responsive human zinc finger gene, MZF-1, preferentially expressed in myeloid cells, J. Biol. Chem. 266 (1991) 14183–14187.
[19] P. Hui, X. Guo, P.G. Bradford, Isolation and characterization of the human gene encoding the myeloid zinc finger protein MZF-1, Biochemistry 34 (1995) 16493–16502.
[20] 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 nonconsensus DNA sequence represses rat GnRH, Mol. Cell. Endocrinol. 117 (1996) 27–39.
[21] J.K. Kepa, C.I. Neeley, B.M. Jacobsen, J.M. Bruder, D.P. McDonnell, K.K. Leslie, M.E. Wierman, Estrogen receptor mediated expression of rat gonadotropin-releasing hormone (GnRH) promoter activity in hypothalamic cells, Endocrine 2 (1994) 947–956.
[22] 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, Nucl. Acids Res. 20 (1992) 1393–1399.
[23] J.M. Lehmann, X.K. Zhang, G. Graupner, M.O. Lee, T. Hermann, B. Hoffmann, M. Pfahl, Formation of retinoid X receptor homodimers leads to repression of T3 response: hormonal cross talk by ligand-induced squelching, Mol. Cell. Biol. 13 (1993) 7698–7707.
[24] P.E. Lovat, M. Annicchiarico-Petruzzelli, M. Corazzari, M.G. Dobson, A.J. Malcolm, A.D. Pearson, G. Melino, C.P. Redfern, Differential effects of retinoic acid isomers on the expression of nuclear receptor co-regulators in neuroblastoma, FEBS Lett. 445 (1999) 415–419.
[25] H.C. Lu, G. Eichele, C. Thaller, Ligand-bound RXR can mediate retinoid signal transduction during embryogenesis, Development 124 (1997) 195–203.
[26] D.J. Mangelsdorf, R.M. Evans, The RXR heterodimers and orphan receptors, Cell 83 (1995) 841–850.
[27] D.J. Mangelsdorf, S.A. Kliewer, A. Kakizuka, K. Umesono, R.M. Evans, Retinoid receptors, Recent Prog., Horm. Res. 48 (1993) 99–121.
[28] A.J. Mason, J.S. Hayflick, R.T. Zoeller, W.S. Young III, H.S. Phillips, P.H. Seeburg, A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse, Science 234 (1986) 1366–1371.
[29] S. Minucci, K. Ozato, Retinoid receptors in transcriptional regulation, Curr. Opin. Genet. Dev. 6 (1996) 567–574.
[30] J.F. Morris, R. Hromas, F.J. Rauscher 3rd, Characterization of the DNA-binding properties of the myeloid zinc finger protein MZF1: two independent DNA-binding domains recognize two DNA consensus sequences with a common G-rich core, Mol. Cell. Biol. 14 (1994) 1786–1795.
[31] J.F. Morris, F.J. Rauscher 3rd, B. Davis, M. Klemsz, D. Xu, D. Tenen, R. Hromas, The myeloid zinc finger gene, MZF-1, regulates the CD34 promoter in vitro, Blood 86 (1995) 3640–3647.
[32] J.L. Napoli, Biochemical pathways of retinoid transport, metabolism, and signal transduction, Clin. Immunol. Immunopathol. 80 (1996) S52–S62.
[33] S.B. Nelson, S.A. Eraly, P.L. Mellon, The GnRH promoter: Target of transcription factors, hormones, and signal transduction pathways, Mol. Cell. Endocrinol. 140 (1998) 151–155.
[34] C.P. Redfern, P.E. Lovat, A.J. Malcolm, A.D. Pearson, Differential effects of 9-cis and all-trans retinoic acid on the induction of retinoic acid receptor-β and cellular retinoic acid-binding protein II in human neuroblastoma cells, Biochem. J. 304 (1994) 147–154.
[35] A.C. Ross, Cellular metabolism and activation of retinoids: roles of cellular retinoid-binding proteins, FASEB J. 7 (1993) 317–327.
[36] S.A. Ross, P.J. McCaffery, U.C. Drager, L.M. De Luca, Retinoids in embryonic development, Physiol. Rev. 80 (2000) 1021–1054.
[37] A. Rowe, Retinoid X receptors, Int. J. Biochem. Cell Biol. 29 (1997) 275–278.
[38] G. Salbert, A. Fanjul, F.J. Piedrafita, X.P. Lu, S.J. Kim, P. Tran, M. Pfahl, Retinoic acid receptors and retinoid X receptor-alpha down-regulate the transforming growth factor-beta 1 promoter by antagonizing AP-1 activity, Mol. Endocrinol. 7 (1993) 1347–1356.
[39] K.D. Sarge, S.P. Murphy, R.I. Morimoto, Activation of heat shock gene transcription by heat shock factor 1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress, Mol. Cell. Biol. 13 (1993) 1392–1407.
[40] P. Sassone-Corsi, Transcriptional checkpoints determining the fate of male germ cells, Cell 88 (1997) 163–166.
[41] I.J. Schippers, M. Kloppenburg, L. Snippe, G. Ab, 9-cis-retinoic acid represses estrogen-induced expression of the very low density apolipoprotein II gene, Mol. Cell. Endocrinol. 105 (1994) 175–182.
[42] R.D. Semba, Vitamin A, immunity and infection, Clin. Infect. Dis. 19 (1994) 489–499.
[43] S.M. Smith, E.D. Dickman, S.C. Power, J. Lancman, Retinoids and their receptors in vertebrate embryogenesis, J. Nutr. 128 (1998) 467S–470S.
[44] B.F. Tate, A.A. Levin, J.F. Grippo, The discovery of 9-cis retinoic acid: A hormone that binds the retinoid X receptor, Trends Endocrinol. Metab. 5 (1994) 189–194.
[45] N.L. Weigel, Y. Zhang, Ligand-independent activation of steroid hormone receptors, J. Mol. Med. 76 (1998) 469–479.
[46] D.B. Whyte, M.A. Lawson, D.D. Belsham, S.A. Eraly, C.T. Bond, J.P. Adelman, P.L. Mellon, A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons, Mol. Endocrinol. 9 (1995) 467–477.