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Cloning and Functional Expression of an E Box-Binding Protein from Rat Granulosa Cells[1]

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ABSTRACT

Ovarian granulosa cells undergo cell growth and cytodifferentiation during follicular maturation. In a number of tissues, the gene expression that is responsible for the cytodifferentiation is largely dependent on E box(es) located upstream of the responsible genes. In this study, we report on the cloning of cDNA(s) encoding E box (5′-CACGTG-3′)-binding protein from a rat granulosa cell cDNA library using a yeast one-hybrid system. When multiple E box sequences were used as target, we obtained a positive clone that encodes the rat homologue of upstream stimulatory factor 2 (USF2). An analysis of the nucleotide sequence and its deduced amino acid sequence reveals that rat USF2 protein consists of 346 amino acid residues and belongs to the basic helix-loop-helix/leucine zipper protein family. Northern blot analysis shows that rat USF2 mRNA exists as multiple forms between 1.6 and 2.2 kilobases. The size of the cloned insert was identical to that of the transcript of maximal length. Electrophoretic mobility shift assays showed that in vitro-translated rat USF2 specifically binds to the E box. In addition, cotransfection experiments with luciferase-reporter constructs in HepG2 cells reveal that the overexpression of rat USF2 leads to an increase of luciferase activity in the E box sequence-dependent manner. Thus, we report molecular cloning, expression, and functional characterization of full-length rat USF2 cDNA.

gene regulation, granulosa cells, ovary

INTRODUCTION

Ovarian granulosa cells play important roles in follicular maturation and ovulation. These functions are regulated by both gonadotropins, FSH, and LH [1–4]. The FSH stimulates growth and differentiation of granulosa cells in the ovary by inducing specific ovarian genes. Granulosa cells undergo sequential cytodifferentiation from small, preantral follicles to large, antral follicles. During such a process, many ovarian genes, such as the LH receptor, P450 side-chain cleavage enzyme, and steroidogenic acute regulatory protein, are induced by gonadotropin stimulation [5, 6]. The expression of the genes is largely dependent on the specific nucleotide sequences upstream from them.

The E box sequence (5′-CANNTG-3′) is found in the transcriptional regulatory region of a number of genes and controls their transcription in biological events including development, cell differentiation, cell growth, and oncogenesis [7]. Transcription factors that contain a basic helix-loop-helix (bHLH) motif, with or without a leucine zipper (LZ) motif, bind to and transactivate transcription from the E box [7]. These proteins consist of two classes: one encompasses ubiquitous transcription factors, such as upstream stimulatory factor (USF) 1, USF2, and Myc; and the other encompasses cell type-specific proteins, including NeuroD/β-cell E-box trans-activator 2, Hairy and Enhancer of Split, MyoD, and myogenin [8–14].

To understand the molecular mechanisms underlying cell type-specific transcription and its hormonal regulation in granulosa cells, we report on a search for and cloning of granulosa cell-specific transcription factor(s). As the first trial, we explored the E box-binding protein using the yeast one-hybrid system. Here, we report molecular cloning of the full-length rat USF2 cDNA, its expression, and its functional characterization.

MATERIALS AND METHODS

Hormones and Materials

Rat FSH (I-8) was obtained from the National Hormone and Pituitary Distribution Program (Bethesda, MD). Diethylstilbestrol (DES) was purchased from Sigma Chemical Co. (St. Louis, MO). The eCG was a product from Teikokuzouki, Inc. (Tokyo, Japan). The Trizol reagent, cDNA synthesis system, Superscript II, and Electromax DH5α were obtained from Life Technologies, Inc. (Grand Island, NY). The oligotex dT-30 super was obtained from Roche Molecular Biochemicals (Indianapolis, IN). Cohesive-end *Eco*RI/*Not*I adapter, T7 Sequenase version 2.0 DNA sequencing kit, and Thermo Sequenase II dye termination cycle sequencing kit were purchased from Amersham Pharmacia Biotech (Cleveland, OH). The yeast one-hybrid system and the pACT2 vector were purchased from Clontech (Palo Alto, CA). The dual luciferase-reporter assay system, pGL3-Basic, pRL-SV40 vectors, and T7 TNT Quick-coupled transcription/translation system were purchased from Promega (Madison, WI). The pcI-neo was purchased from Invitrogen (Carlsbad, CA). The Qiagen plasmid kit was purchased from Qiagen (Hilden, Germany). The BcaBest DNA labeling kit was purchased from Takara Shuzo (Kyoto, Japan). The α-^{32}P-deoxycytidine triphosphate (dCTP; 111 TBq/mmol) and γ-^{32}P-ATP (111 TBq/mmol) were obtained from NEN Life Science Products (Wilmington, DE).

Animals and Treatment

Immature, Kwl:Wistar female rats (21 days old) were used. The rats were treated with 2 mg of DES in 0.1 ml of sesame oil once daily for 4 days for preparation of granulosa cells. For in vivo study, 30 IU of eCG were administrated, and ovaries were removed at the indicated times. At

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all times, the animals were treated according to National Institutes of Health guidelines.

Cells and Cell Culture

Rat granulosa cells were isolated from DES-treated, immature female rat ovaries and cultured as described elsewhere [15]. The HepG2 cells, a human hepatoma cell line, were purchased from the American Type Cell Collection (Manassas, VA) and cultured as described elsewhere [16].

Preparation and Analysis of RNA

Total RNA was prepared from granulosa cells that had been treated with rat FSH (30 ng/ml) for 90 min and eCG-primed, immature rat ovaries using the Trizol reagent. Total RNA (10 μg/lane) was subjected to a 1% v/v denaturing agarose gel electrophoresis and then transferred to a Biodyne membrane (ICN Biomedicals, Inc., Glen Cove, NY). The filter was prehybridized in 50% formamide, 5× SSC (1×: 0.15 M sodium chloride and 0.015 M sodium citrate), 5× Denhardt, 0.5% SDS, and 200 μg/ml of salmon sperm DNA at 42°C for 16 h and then hybridized with a ^{32}P-labeled probe for 16 h. After washing at 60°C in 0.1× SSC and 0.1% SDS, the filter was exposed at −80°C with an intensifying screen to a Kodak X-AR film (Eastman Kodak Co., Rochester, NY) or a Fujix imaging plate (Kanagawa, Japan). Hybridization signals were detected by autoradiography or with the Fujix BAS-1500 image analyzing system.

The USF2 (5′-GGAGGCCAGGTGACATACCGCGTA-GTCCAGGTG-3′) and ASUSF2 (5′-CACCTGGACTAC-3′) oligonucleotides were annealed to form a double-stranded oligonucleotide, which was then labeled with the α-^{32}P-dCTP by Klenow reaction. The rat 18S rRNA gene was a gift of Dr. Tamio Noguchi (Nagoya University, Japan). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and 18S rRNA gene were labeled with α-^{32}P-dCTP using the BcaBest DNA labeling kit.

Construction of a cDNA Library

Total RNA was prepared from granulosa cells that had been treated with FSH (30 ng/ml) for 90 min, and poly(A)$^+$-RNA was isolated with oligotex dT-30 super. Complementary DNA was synthesized from 15 μg of poly(A)$^+$-RNA with the cDNA synthesis system and Superscript II. After an *Eco*RI/*Not*I adapter was added to both ends of the cDNA, it was subjected to size-fractionation with agarose gel electrophoresis. Finally, the resultant cDNA was ligated into the *Eco*RI site of the pACT2 and electroporated to Electromax DH5α. The cDNA library contained 1×10^7 independent clones.

One-Hybrid System

Oligonucleotides used in this system were as follows: H/K mcs, 5′-AGCTTGAATTCAGATCTTCTAGAGGT-AC-3′; K/H mcs, 5′-CTCTAGAAGATCTGAATTCA-3′; E boxs, 5′-GATCAGTTCTCACGTGGTGGCCACGTGCTTG-3′; E boxas, 5′-GATCCAAGCACGTGGCCACCACGT-GAGAACT-3′; Xba E boxs, 5′-CTAGAGTTCTCA-CGTGGTGGCCACGTGCTTG-3′; Xba E boxas, 5′-CTA-GCAAGCACGTGGCCACCACGTGAGAACT-3′; Xba mut E boxs, 5′-CTAGAGTTCTCA*TGC*GGTGGCC*GC*ATGCT-TG-3′; and Xba mut E boxas, 5′-CTAGCAAGCA-TGCGGCCACCGCATGAGAACT-3′. The H/K mcs and K/H mcs oligonucleotides were annealed, phosphorylated, and ligated into the *Hin*dIII/*Kpn*I sites of the pLacZi plasmid to obtain the pLacZiB1 plasmid. The E box and E boxas oligonucleotides were annealed, phosphorylated, and ligated into the *Bgl*II site of the pLacZiB1 plasmid to produce the 3× E box-LacZ reporter plasmid. This was also subcloned into the *Bam*HI site of the pGEM3. A *Sac*I/*Xba*I fragment from the pGEM3 vector, which harbored three copies of the double-stranded oligonucleotide, was isolated and ligated into the *Sac*I/*Spe*I sites of the pRW95-1 to produce the 3× E box-HIS3 reporter plasmid. The pRW95-1 was a gift from Dr. M. Schweizer [17].

The YM4271 yeast cells were sequentially transformed with both reporter plasmids. The reporter yeast strain was transformed with a rat granulosa cell cDNA library using a high-efficiency transformation method [18]. When 6.8×10^6 clones were screened, one positive clone was obtained. A plasmid, pE6, was isolated from the yeast, and its nucleotide sequence was determined.

Plasmids

The tkCAT vector was a gift from Dr. Daryl K. Granner (Vanderbilt University, Nashville, TN). The tkCAT was digested with *Bam*HI, and a 164-base pair (bp) insert DNA was subcloned into the *Bgl*II site of the pGL3-Basic vector to produce the tk/Luc plasmid. The Xba E box and Xba E boxas as well as the Xba mut E box and Xba mut E boxas oligonucleotides were annealed, phosphorylated, and ligated into the *Nhe*I site of the ptk/Luc plasmid, respectively. These plasmids are hereafter referred to as E box-tk/Luc and mut E box-tk/Luc, respectively. The pE6, a plasmid cloned using the one-hybrid system, was digested with *Eco*RI, and a 2.2-kilobase (kb) fragment was subcloned into the *Eco*RI site of the pcI-neo plasmid to obtain the pcI-neo/USF2. The sequences of all plasmids were confirmed by dideoxy sequencing.

In Vitro-Translation and Electrophoretic Mobility Shift Assays

One microgram of the pcI-neo or pcI-neo/USF2 plasmid was incubated at 30°C for 60 min with the T7 TNT Quick-coupled transcription/translation system in the presence of methionine. The in vitro-synthesized product was then subjected to electrophoretic mobility shift assays (EMSAs), which were performed as described elsewhere [19] with minor modifications. Briefly, one μl of one-third-diluted, in vitro-translated product was added to the binding mixture with a ^{32}P-labeled probe. For a competition analysis, a 200-fold molar excess of competitor DNAs was added to the binding mixture. After completion of the binding, the mixture was subjected to electrophoresis on a 6% polyacrylamide gel (19:1 = acrylamide:bis-acrylamide) in 44.5 mM Tris-HCl (pH 8.0), 44.5 mM boric acid, and 1 mM EDTA at 200 V for 1 h, after which the gels were dried and exposed at −80°C for 16 h to Kodak X-AR film.

DNA Transfections and Luciferase Assays

All plasmids used for transfection were prepared with the Qiagen plasmid kit, followed by CsCl gradient ultracentrifugation. The DNA transfections for HepG2 cells have been described elsewhere [16]. The luciferase-reporter plasmid (300 ng), pRL-SV40 (6 ng), and expression plasmid (100 ng) were used for transfection. Four hours after transfection, the medium was changed. The cells were harvested for determination of both firefly and sea pansy lu-

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  1  MDMLDPGLDP ASSATAAAAA SHDKGPEAEE GVELQEGGDG PGAEEQTAVA   50

 51  IASVQQAAFG DHNIQYQFRT ESNGGQVTYR VVQVTDGQLD GQGDAAGAVS  100

101  VVSTAAFAGG QQAVTQVGVD GAAQRPGPAA ASVPTGPAAP FPLAVIQNPF  150

151  SNGGSPAAEA VSGEARFAYF PASSVGDTTA VSVQTTDQSL QAGGQFYVMM  200

201  TPQDVLQTGT QRTIAPRTHP YSPKIDGTRT PRDERRRAQH NEVERRRRDK  250

251  INNWIVQLSK IIPDCHADNS KTGASKGGIL SKACDYIREL RQTNQRMQET  300

301  FKEAERLQMD NELLRQQIEE LKNENALLRA QLQQHNLEMV GESTRQ      346
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FIG. 1. Deduced amino acid sequence of rat USF2. The amino acids shown on both sides are numbered starting from the initiation codon. The bHLH region and LZ are indicated by underlining and asterisks, respectively.

ciferase activities at 48 h after transfection. Firefly and sea pansy luciferase assays were carried out according to the manufacturer's recommended protocol. Luciferase activities were determined by Lumat LB9501 luminometer (Berthold, Germany). Firefly luciferase activities (expressed as relative light units) were normalized relative to sea pansy luciferase activities.

RESULTS

An attempt was made to clone the cDNA(s) for the E box-binding protein using the yeast one-hybrid system. Six copies of E box sequence (5′-CACGTG-3′) were inserted upstream of the *gal1*-minimal promoter of the HIS3- or LacZ-based reporter plasmids. Using a yeast harboring both reporter plasmids, 6.8×10^6 of a rat granulosa cell cDNA library was screened. We obtained a HIS3- and LacZ-double-positive clone, E6. The nucleotide sequence of the E6 was determined, and a search for similar sequences was made in the GenBank database. The results showed that it was similar to the nucleotide sequence of human, mouse, and rat USF2 cDNAs. Full-length nucleotide sequences of the human and mouse USF2 cDNAs have been reported [20–23]. However, only a partial sequence of rat USF2 is registered in the database, and the characterization of entire rat USF2 protein as well as the gene is largely unknown [23]. We then determined the nucleotide sequence of the full-length rat USF2 cDNA and characterized the corresponding protein (nucleotide sequence of the USF2 cDNA was submitted to DNA Data Bank of Japan, accession no. AB047556). The amino acid sequence of rat USF2, as deduced from its nucleotide sequence, is shown in Figure 1. Rat USF2 protein consists of 346 amino acid residues and belongs to the bHLH/LZ protein family.

We next examined the expression and regulation of rat USF2 mRNA. As shown in Figure 2A, Northern blot analysis revealed that rat USF2 mRNA was detected as multiple bands between 1.6 and 2.2 kb in granulosa cells. Because the size of our cloned insert was 2171 bp, it is identical with the transcript of maximal length. Administration of eCG to the immature female rat leads to the promotion of follicular development and differentiation. The issue of whether the level of USF2 mRNA is altered in eCG-primed, immature rat ovary was examined. As shown in Figure 2B, the level of USF2 mRNA was not altered within 48 h after eCG treatment.

To characterize the function of rat USF2 protein, we employed two independent methods. First, the Xba E box oligonucleotide was employed as the probe in EMSAs. Two plasmids, the pcI-neo and pcI-neo/USF2, were prepared. The pcI-neo is an empty vector, and the pcI-neo/USF2 is a vector in which the full-length rat USF2 cDNA is inserted directionally just downstream of a T7 promoter in the pcI-neo. The RNAs can be synthesized from the T7 promoter in the presence of T7 RNA polymerase using these plasmids. These were subjected to a T7-based, in vitro-transcription/translation system. When the ^{32}P-labeled probe was incubated with reticulocyte lysates that had been programmed with the pcI-neo, no DNA-protein complex was detected (Fig. 3). In contrast, when the probe was incubated with in vitro-synthesized USF2, a DNA-protein complex was observed. In competition experiments, the formation of this complex was prevented by the addition of a 200-fold molar excess of the unlabeled Xba E box oligonucleotide, but not by an equimolar amount of Xba mut E box oligonucleotide, which is made by replacing a core of E box sequence CACGTG with CATGCG. These results indicate that in vitro-translated USF2 specifically binds to the E box.

Finally, to determine the transcriptional role of USF2,

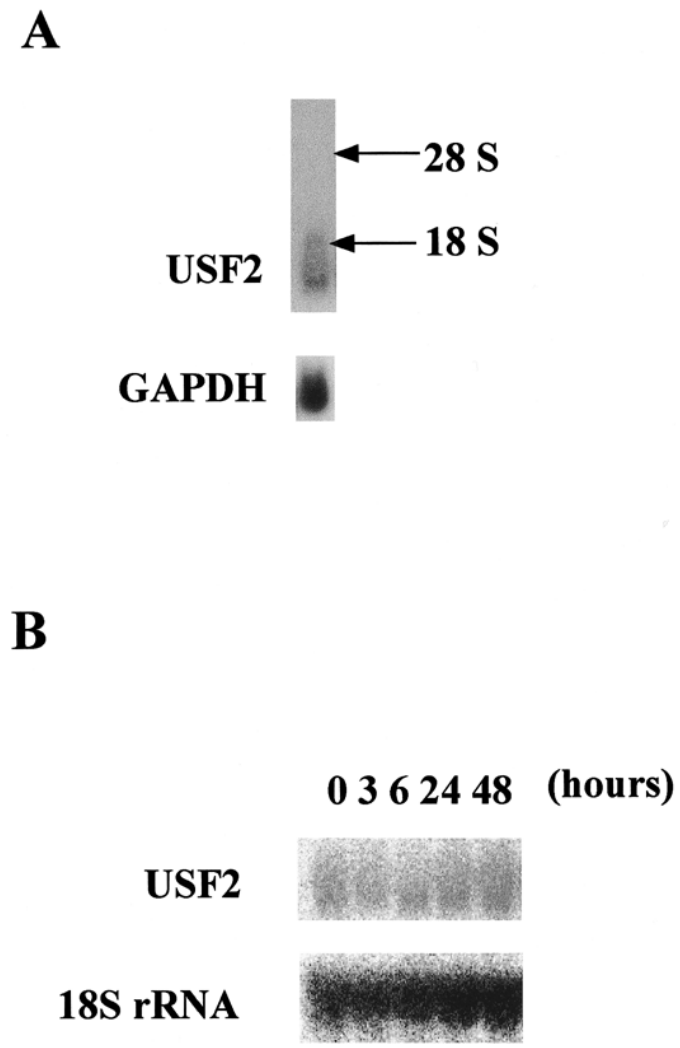

FIG. 2. Northern blot analysis of rat USF2 mRNA. Ten micrograms of total RNA from rat granulosa cells (**A**) or rat ovaries treated with eCG for various times (**B**) were subjected to 1% denaturing agarose gel electrophoresis and then transferred to a nylon membrane. The membranes were hybridized with ^{32}P-labeled USF2, GAPDH, or 18S rRNA probes. Size markers are shown on the left.

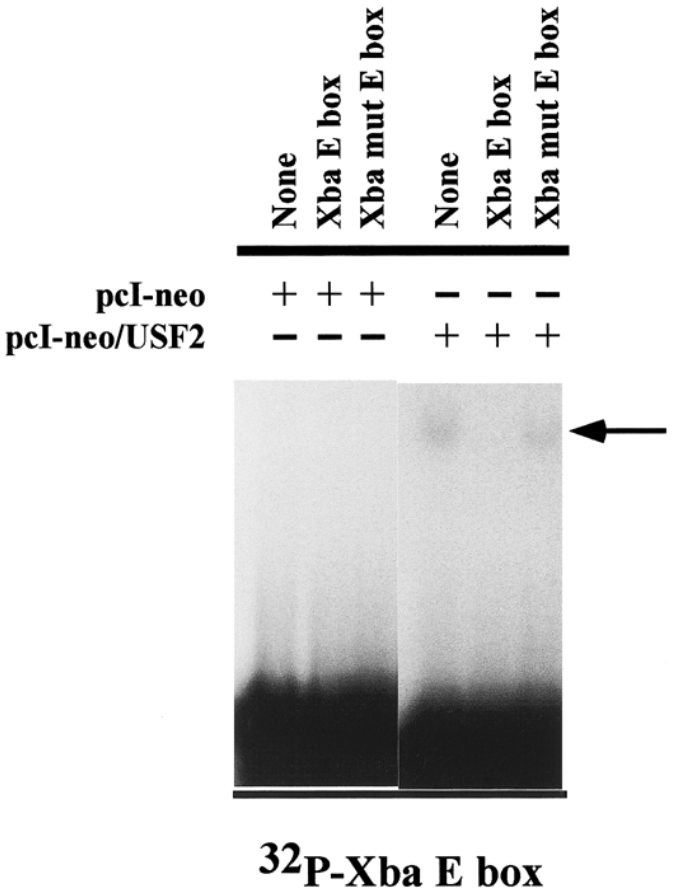

FIG. 3. In vitro-translated USF2 protein specifically binds to the E box sequence. The pcI-neo or pcI-neo/USF2 was mixed with reticulocyte lysates. The product was incubated with a ^{32}P-labeled, double-stranded, Xba E box oligonucleotide, with or without a 200-fold molar excess of competitor DNAs, and then subjected to native polyacrylamide gel electrophoresis. The arrow indicates a binding complex. Programmed lysates and competitor DNAs are shown at the top.

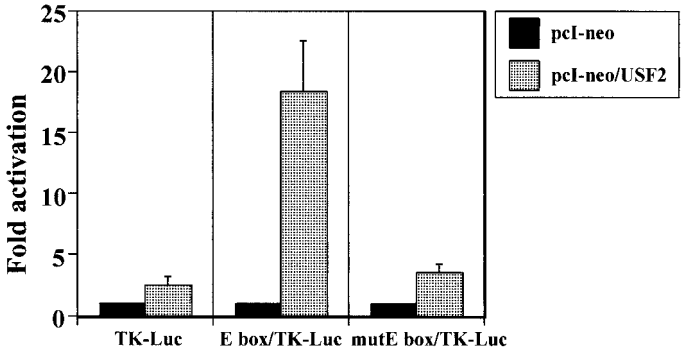

FIG. 4. USF2 stimulates transcription of the reporter plasmid in the E box-dependent manner. Reporter plasmid (300 ng), pRL-SV40 (6 ng), and expression vector (100 ng) were transfected into HepG2 cells using a calcium phosphate method. Forty-eight hours after transfection, both firefly and sea pansy luciferase activities were determined. The normalized firefly luciferase activity from each reporter plasmid cotransfected with the pcI-neo was defined as 1. Each column and bar represent the mean and SEM, respectively, of at least three independent transfection experiments.

cotransfection experiments were performed. Three reporter plasmids were used: tk/Luc, E box-tk/Luc, and mut E box-tk/Luc. The tk/Luc contains a thymidine kinase promoter linked to the firefly luciferase gene. The E box-tk/Luc and mut E box-tk/Luc harbored three copies of Xba E box and mut Xba E box oligonucleotides just upstream of the tk promoter, respectively. The luciferase activity of each reporter plasmid in the presence of the pcI-neo is normalized relative to a value of 1.0. When the tk/Luc reporter plasmid was cotransfected with the pcI-neo/USF2 into HepG2 cells, luciferase activity increased by 2.5-fold (Fig. 4). In contrast, when the E box-tk/Luc was cotransfected with the pcI-neo/USF2, the luciferase activity was dramatically enhanced by 18.4-fold. However, the introduction of a mutation in the E box sequence decreased the luciferase activity by 3.5-fold. These results suggest that USF2 stimulates transcription in the E box sequence-dependent manner.

DISCUSSION

The full-length rat USF2 cDNA from a granulosa cell cDNA library was cloned using the yeast one-hybrid system and then characterized. The USF was originally identified as a transcription factor that binds to the adenovirus major late promoter and was purified from HeLa cells [9]. Purified USF was found to exist in multiple forms, 43 kDa (i.e., USF1) and 44 kDa (i.e., USF2), on an SDS-polyacrylamide gel [9]. The full-length human USF1 cDNA has been cloned and shown to belong to the bHLH/LZ family [24]. At first, partial USF2 cDNA was cloned as the c-Fos-interacting protein [20]. After full-length human and mouse USF2 cDNAs had been cloned, it was shown that both USF1 and USF2 could form heterodimers as well as homodimers [22, 23]. In addition, two isoforms of human USF2 protein can be produced by alternative RNA splicing. Both isoforms, USF2a and USF2b, function as transcriptional activators and encode for 346 and 269 amino acid residues, respectively [23]. Because our cloned rat USF2 contains 346 amino acid residues, it would be predicted to be the USF2a form. The homology of the amino acid sequence of rat USF2 with human or mouse USF2 protein was 98% and 100%, respectively. The cloned insert contains 2171 bp, and the size is same as the largest transcript of USF2 as determined by Northern blot analysis. It is likely that smaller-size mRNAs might contain USF2b mRNA. The EMSAs showed that in vitro-translated USF2a protein can specifically bind to the E box sequence in vitro. In addition, cotransfection of USF2a protein expression vector with reporter plasmids showed that it caused an enhancement of luciferase activity in the E box-dependent manner. Thus, we conclude that our cloned cDNA encoded functional, bona fide USF2a rat counterpart.

In Northern blot analysis, β-actin or GAPDH mRNAs have been used as internal controls in most studies. However, under some conditions, such as treatment with hormones or growth factors, expression of these mRNA is not reliably steady state. We found that USF2 mRNA is also a promising candidate as an internal control in the ovary, because its level was not altered by the presence of gonadotropins.

The Ad4-binding protein/steroidogenic factor-1 (Ad4BP/SF-1) is a transcription factor that regulates the expression of the steroidogenic genes and sexual differentiation [25, 26]. An E box sequence in the promoter region is critical for the cell type-specific expression in the mammalian Ad4BP/SF-1 genes [27, 28]. It has been reported that USF is a key regulator of the E box-binding protein [29]. Prostaglandin endoperoxide synthase (PGS)-2, also referred to as cyclooxygenase-2, is a rate-controlling enzyme in the biosynthetic pathway of prostaglandins from arachidonic acid

[30]. The PGS-2 is selectively induced by gonadotropins in granulosa cells [5]. In PGS-2-deficient mice, females become infertile because of ovulation failure and certain impaired reproductive processes [31]. An analysis of the regulatory elements of the rat PGS-2 gene promoter in primary cultured granulosa cells has revealed that an E box is essential for the promoter activity, and that USF binds to the element [32]. In addition, it was recently reported that both USF1 and USF2 proteins regulate transcription of the FSH-receptor gene in granulosa cells [33]. Our data clearly indicate that the level of USF2 mRNA is not altered by gonadotropins. The level of USF2 protein also is not changed by treatment with gonadotropins [32], and it has been reported that the transcriptional activity of USF1 is regulated in a redox- and phosphorylation-dependent manner, and that unidentified HeLa cell-specific coactivator(s) may interact with both USF1 and USF2, thus regulating their activities [34–36]. To explain the involvement of an E box sequence in granulosa cell-specific expression of these genes and their regulation by gonadotropins, an analysis of the modifications of both USF1 and USF2 proteins, identification and characterization of their interacting protein, or a further search for another E box-binding factor is required.

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