An E-box Region within the Prostaglandin Endoperoxide Synthase-2 (PGS-2) Promoter Is Required for Transcription in Rat Ovarian Granulosa Cells*

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The prostaglandin endoperoxide synthase-2 (PGS-2) gene encodes an isoform of prostaglandin synthase that is transiently induced by protein kinase A (luteinizing hormone) and protein kinase C (gonadotropin-releasing hormone) agonists in granulosa cells of ovolating follicles. The promoter of the rat PGS-2 gene contains a CAAT enhancer-binding protein consensus site (CAAT box) which can confer hormone inducibility to a PGS-2 CAT reporter gene, as well as a putative E-box region. To determine if the E-box region was involved in hormone induced trans-activation of the rat PGS-2 gene, constructs with the CAAT box and E-box regions (−192 PGS-2 CAT), only the putative E-box (−110 PGS-2 CAT), or neither region (−52 PGS-2 CAT) were transiently transfected into rat granulosa cell cultures. CAT activity was induced in both the −192 and −110 PGS-2 CAT vectors by luteinizing hormone (10-fold) and gonadotropin-releasing hormone (6-fold), whereas CAT activity of the −52 PGS-2 CAT construct did not differ from the promoterless vector (pCAT-Basic). Deletion of 1 base pair from the E-box within the −110 PGS-2 CAT construct, as well as point mutations within the CAAT box, E-box, or both regions of the −192 PGS-2 CAT construct, demonstrated that the E-box is critical for basal transcription, and that regions, in addition to the CAAT box, are involved in hormone induction of the PGS-2 gene. An oligonucleotide spanning the rat PGS-2 E-box bound two specific protein complexes which were supershifted in the presence of antibody specific for the upstream stimulatory factor. Thus, in rat granulosa cells, the PGS-2 E-box region appears to interact with upstream cis-acting elements other than the CAAT box to confer hormonal regulation of the gene. The E-box region of the rat PGS-2 promoter does not contain ATF/CRE activity found in the human and mouse PGS-2 promoters, but is critical for basal transcription of the PGS-2 gene in rat granulosa cells and binds the upstream stimulatory factor (as do E-box regions of other genes regulated in the ovary).

Prostaglandin endoperoxide synthase (PGS) catalyzes the rate-limiting step of prostaglandin biosynthesis. The synthase is a homodimer with two subunits and has two catalytic properties, a cyclooxygenase activity that is required for the conversion of arachidonic acid to prostaglandin G2, and a peroxidase activity that reduces prostaglandin G2 to prostaglandin H2. Prostaglandins are associated with inflammatory responses, wound healing, bone development, glomerular filtration and water balance, and hemostasis (for a review, see Ref. 2). Prostaglandins have also been shown to regulate a variety of reproductive functions, including ovulation (3) and parturition (4). For example, ovulation is inhibited by aspirin, indomethacin, and specific inhibitors of PGS, in mice (5), rats (6–8), rabbits (9, 10), and pigs (11). Most recently, targeted deletion of the PGS-2 gene has been shown to prevent ovulation in mice (12), whereas targeted disruption of the PGS-1 gene did not alter ovarian function, but did compromise parturition (13).

Two isoforms of the PGS enzyme are present in the rat ovary (14, 15). Each enzyme is the product of a distinct gene as evidenced by the cloning of cDNAs for PGS-1 (16–18) and PGS-2 (19–21). PGS-1 is encoded by a 2.8-kilobase pair RNA transcript and the enzyme is ubiquitously expressed in theca cells of the ovarian follicle (15), as well as other tissues (22). PGS-2 is encoded by a 4.4-kilobase pair RNA transcript and is rapidly induced in ovarian granulosa cells by luteinizing hormone (LH), follicle-stimulating hormone (FSH), forskolin, gonadotropin-releasing hormone (GnRH), and epidermal growth factor (14, 15, 23). The presence of PGS-2 mRNA in granulosa cells corresponds to the specific immunological localization of this protein in granulosa cells, not theca cells (14, 15, 24). PGS-2 mRNA is rapidly and transiently induced in the ovary (25) and other tissues by activators of several signaling pathways including protein kinase A, protein kinase C, and tyrosine kinase pathways (22, 23, 26, 27).

The 5'-flanking region of the mouse (28, 29), chicken (30), rat (31), and human (32) PGS-2 genes have been isolated, sequenced, and shown to contain numerous regions of homology (Fig. 1), including putative binding sites for these transcription factors: NF-kB, Sp1, AP-2, CAAT/enhancer binding protein (C/EBP), CAMP response element binding protein (CREB), and E-box binding proteins. Deletion analyses of the promoters have identified regions of functional trans-activation between −195/32 for the rat (31), between −140/−132 and −124/−52 for...
the human (33, 34), and between −512/−385, −186/−131 and −80/−32 for the mouse (35–37).

The functional region of the mouse PGS-2 promoter contains NFκB, C/EBP, and an overlapping ATF/CRE (5′-CGCTA-3′) and E-box (5′-CACGTG-3′) transcription factor recognition sequences (35–37). Mutational analysis of the NFκB and C/EBP elements demonstrated that both these sites were important for tumor necrosis factor-α induction of PGS-2-luciferase constructs transfected into MC3T3-E1 osteoblastic cells (35). Mutational analysis of the ATF/CRE element within the mouse promoter demonstrated that the ATF/CRE element, but not the E-box element, is essential for v-erbA induction of PGS-2-luciferase constructs transfected into NIH 3T3 fibroblasts (36). Although the E-box was not required for v-erbA activation of the PGS-2 expression vectors (36), mutations of the E-box reduced basal levels of transcription (36). Electrophoretic mobility shift assays demonstrated that an end-labeled mouse PGS-2 oligonucleotide (−65/−39 bp), containing ATF/CRE and E-box elements, bound several fibroblast extract proteins which by competition assays and antibody analyses were identified as CREB, c-jun, and two E-box-binding proteins (36, 37). Furthermore, c-jun, and not CREB, was shown to be required for v-erbA induction of PGS-2 promoter constructs in NIH 3T3 fibroblasts (37).

When human PGS-2 promoter reporter vectors were transfected into bovine arterial endothelial cells (33) or human U937 monocyteic cells (34), various regions (NFκB, CAAT box (C/EBP), and ATF/CRE) were shown to be important for the expression of transgenes dependent upon the cell type. Although deletion to −52 disrupted the putative human PGS-2 E-box and abolished functional activity of the human PGS-2 transgenes, specific functional analysis of this element in the context of other regions has not yet been reported. In addition, the human PGS-2 E-box element is different by one base pair from the E-box element present in the rat and mouse PGS-2 promoters (see Table I). Therefore, the human E-box element might bind different E-box binding proteins, than those which bind the rat or mouse PGS-2 E-box element.

In the 5′-flanking region of the rat PGS-2 gene, a CAAT box region (−140/−132) has been identified and shown to confer cAMP-responsiveness when ligated to a minimal −52/32 PGS-2CAT reporter construct (38). Mutation of this CAAT box region decreased cAMP inducibility of the fusion construct in rat ovarian granulosa cells by 50%. The CAAT box binds to C/EBPα and C/EBPβ proteins present in nuclear extracts of rat granulosa cells (38). Furthermore, LH induces C/EBPβ mRNA and decreases C/EBPα mRNA coordinately with the induction of the PGS-2 gene (38). However, because the PGS-2-CAT fusion vectors used in these experiments disrupted what has recently been characterized as a CRE/ATF and E-box regulatory region in the mouse promoter, additional analyses were required to determine the specific role of each region for transactivation of the rat PGS-2 promoter in ovarian granulosa cells (38).

Based on the evidence that multiple factors bind the ATF/CRE, E-box and CAAT elements of the PGS-2 gene, and that these regions confer trans-activation in an agonist, cell-type, or species promoter-specific manner, we have designed experiments to determine if the ATF/CRE or E-box region of the rat PGS-2 promoter might also be functionally relevant for transactivation of the rat PGS-2 gene by LH and GnRH in granulosa cells. Transient transfection of reporter constructs in rat granulosa cells demonstrated that the E-box element is critical for basal activation of the PGS-2 gene in granulosa cells and that one transcription factor which binds to this region is a cAMP-responsive element in the proximal (−110/−52) region of the rat PGS-2 promoter.

**EXPERIMENTAL PROCEDURES**

**Animals—I**mmature female rats (day 23 of age; weight, 55–60 g) were injected with 17β-estradiol (1.5 mg/day for 3 days) beginning on day 24 of age (25, 39, 40). Hypophysectomized immature female rats (day 26 of age) were obtained from Harlan (Indianapolis, IN) 1 day after surgery. Animals were primed with 17β-estradiol (E; 1.5 mg/day subcutaneously for 3 days) and FSH (F; 1.0 µg subcutaneously, twice daily for 2 days) designated HE or HEF, respectively (23). Animals were treated in accordance with the NIH Guide for the Care and Use of...
Cloning—The rat −110 PGS-2-CAT fusion construct was created by ligating a double-stranded −110/−3 oligonucleotide (5′-AGG TGG GGG GTG GGG GTG GGG AAA GAC GGC GAG GAA ACA AGT CAC CAA GTC AC-3′) to the −52/32 PGS-2-CAT construct (38). The latter was produced by digesting a −62/32 PGS-2-CAT construct with HindIII and BclI. The −110 mutant (Mt) PGS-2-CAT fusion construct was generated by ligating a −110/−3 oligonucleotide (5′-AGG TGG GGT GGG GTG GGG GTG GGG AAA GAC GGC GAG GAA ACA AGT CAC CAA GTC AC-3′) into the −52/32 PGS-2-CAT construct. The −110 mutant (Mt) PGS-2-CAT construct was then used as a template to substitute the −15/−31 region of the −52/32 PGS-2-CAT construct with the −110/−3 oligonucleotide (5′-AGG TGG GGT GGG GTG GGG GTG GGG AAA GAC GGC GAG GAA ACA AGT CAC CAA GTC AC-3′). This created a −1-base pair deletion within the putative E-box element (CAGCGT to CA GTG).

Three additional mutations of the PGS-2 promoter were created using the −195/32 PGS-2-CAT construct as a template, −195/32 (C/EBP) CAT, −195/32 (E-box) CAT and a −195/32 (Double Mt) PGS-2-CAT. The −C/EBP Mt was created by substituting four base pairs within the CAT box that disrupts the binding of C/EBP and β protein. In EMSA, the C/EBP mutant oligonucleotide did not compete for the binding of C/EBPs to a labeled −195/−110 DNA fragment (25). The −E-box mutant was created with an oligonucleotide containing a two base pair substitution which also fails to compete for the binding of proteins to a labeled wild type −64/−41 oligonucleotide (see Fig. 3A, lane 7). Finally, the Double Mt includes these same mutations within both the CAT box and the E-box.

The mutations (indicated by lowercase) within the −195 to 32 base pairs of the PGS-2 promoter were created by the polymerase chain reaction (PCR) using mutant oligonucleotides to the CAT box (5′-GTT ATT CCC aTG GAA GC-3′ and 5′-GCT GCC atg gsa ATa ATA CC-3′) and the E-box (5′-AGT CAC GAA GTC A TG AGT CCA-3′ and 5′-AAG TGG ACT TGC ATa (hCG, 10 IU) designated HEF that would hybridize at each end of the promoter region to be cloned (5′-GGC AAG CCT GGC TTC TC-3′ and 5′-TGC TCT AGa GCC TAC GT-3′), external primers that would hybridize at each end of the promoter region to be cloned (5′-GGC AAG CCT GGC TTC TC-3′ and 5′-TGC TCT AGa GCC TAC GT-3′).}

RESULTS

An E-box cis-Acting DNA Element Is Required for Transcriptional Activation of rPGS-2 Promoter in Rat Ovarian Granulosa Cells—Previous functional analyses of rat PGS-2-CAT promoter expression vectors in primary ovarian granulosa cells demonstrated that −110 base pairs of the proximal promoter were sufficient to confer LH, FSH, and forskolin inducibility to the PGS-2-CAT vectors (38). A CAAT box element within this region (−195/−110) was shown to bind C/EBP proteins, specifically C/EBPα and C/EBPβ, and to confer cAMP (FSH, LH, and forskolin) inducibility if ligated to a minimally active −52/32 PGS-2-CAT construct (38). Transfection of the (−192/−110) (−52/32) PGS-2-CAT fusion construct exhibited a decrease in basal transcription (38). Based on recent reports, this construct disrupted the ATF/CRE and/or E-box region (−110/−39) (33, 36).

To determine the functional activity of the −110/−53 region of the rat PGS-2 promoter, we have made additional constructs by ligating a −110/−53 oligonucleotide onto the −52 PGS-2-CAT fusion construct. A mutant construct (−110 mt PGS-2CAT) with a 1-base pair deletion within the E-box region (CAGCGT to CA GTG) was also created. The −110, −110 Mt, and −52 PGS-2-CAT constructs were transiently transfected into differentiated granulosa cells and exposed to LH (Fig. 2A).

All values were measured within the linear range of the assay and then adjusted to the relative CAT activity for
25 μg of protein extract. The vector pCAT-Control (Promega, Madison, WI), which contains the simian virus 40 promoter and enhancer, was utilized in each transfection experiment as a control for transfection efficiency. The pCAT-Control vector routinely displayed a relative CAT activity of 80 ± 10 indicating that transfection efficiency was reproducible among all experiments. The -195 PGS-2-CAT vector exhibited significantly higher relative basal activity than pCAT-Basic, which lacks a promoter. This was reduced 33% with the -110 PGS-2-CAT construct, which lacks the CAAT box element (−142/−139). Basal activity decreased an additional 40% by mutation of the E-box. The relative CAT activity stimulated by LH was 88.7 ± 18 and 30 ± 10 relative activity for the -195 and -110 PGS-2-CAT constructs, respectively. However, the relative CAT activity of the -110 Mt vector induced by LH, was not different from the -52 PGS-2-CAT construct, 5.5 ± 3 and 3.6 ± 0.2, respectively. Thus, by disrupting the E-box region, basal, as well as LH-induced, trans-activation of the -110 PGS-2-CAT reporter construct was reduced in differentiated granulosa cell cultures. Collectively, these data indicate that the E-box region is a functional cis-acting DNA element within the rat PGS-2 promoter that is essential for transcriptional activation of the PGS-2 gene in granulosa cells.

Previous studies have demonstrated that two distinct signaling pathways induce PGS-2 mRNA and protein in preovulatory granulosa cells (23, 26, 27). One pathway involves LH activation of protein kinase A; the other involves GnRH activation of protein kinase C (27). In order to determine if the regions of the rat PGS-2 promoter, that are activated by LH, are also activated by GnRH, the -195, -110, -110 Mt, and the -52 PGS-2-CAT constructs were transiently transfected into differentiated granulosa cells and exposed to GnRH (1 μM; Fig. 2B). The relative basal level of CAT activity measured in granulosa cells transfected with pCAT-Basic was minimal and was not altered.
by addition of GnRH to the cultures. Addition of GnRH increased CAT activity produced by the −195 PGS-2 construct 3-fold, a level of CAT activity approximately one-third of that induced by LH. Relative CAT activity of the −110 PGS-2 CAT construct induced by GnRH did not significantly decrease compared to the −195 PGS-2 CAT construct, while the fold induction of −110 PGS-2 CAT by GnRH increased from 3- to 6-fold due to a decrease in the basal activity of the −110 PGS-2 CAT construct. Disruption of the E-box cis-element within constructs, −110 Mt and −52 PGS-2 CAT, decreased GnRH induction 50%, when compared to the nonmutated −110 PGS-2 CAT promoter construct. These data support the obligatory role of the E-box element for trans-activation of the PGS-2 gene by these two distinct signaling pathways in granulosa cells.

Binding of Protein Complexes to the E-box is Not Determined by the Stage of Follicular Development—As mentioned above, the mouse PGS-2 promoter contains an ATF/CRE and E-box region that is activated in NIH 3T3 cells by cotransfection with a v-src expression plasmid (36). Mutation of these elements within the promoter demonstrated that the ATF/CRE element, but not the E-box, is important for v-src trans-activation of the mouse PGS-2 promoter in NIH 3T3 cells (36). Although the E-box cis-element did not appear to be involved in the v-src induction of the PGS-2 gene in NIH 3T3 cells, a decrease in basal transcription was seen when the E-box element was mutated (36).

To determine if similar, or the same proteins, might bind to the E-box region (−67/−41) of the rat PGS-2 promoter, nuclear extracts were prepared from preovulatory granulosa cells induced with an ovulatory dose of hCG for 2 h; a treatment known to induce expression of the endogenous PGS-2 gene (25). When nuclear extract protein (1 μg) was incubated with an end-labeled −67/−41 oligonucleotide containing the rat PGS-2 promoter E-box element, two protein-DNA complexes were formed (designated I and II; Fig. 3A). The binding reaction was specific as demonstrated by the ability of 10-, 50-, and 100-fold molar excess of unlabeled −67/−41 oligonucleotide to inhibit complex formation (Fig. 3A, lanes 3–5). Additionally, 100-fold excess of an oligonucleotide 5′ of the E-box region (−110/−54) was unable to block the formation of the two protein-DNA complexes (Fig. 3A, lane 6). The oligonucleotide mutated within the E-box region (−67/−41 Mt; CACGTG to CACtTt) also did not prevent complex formation (Fig. 3A, lane 7). These results indicated that granulosa cell nuclei contained protein(s) capable of binding specifically to an E-box element.

To determine if the E-box binding protein(s) present in the nuclear extract of granulosa cells was hormonally regulated during follicular development, the end-labeled −67/−41 oligonucleotide was incubated with nuclear extracts prepared from granulosa cells at different stages of follicular development. Nuclear extracts were collected from granulosa cells of preantral/small antral follicles (HE), preovulatory follicles (HEF), and from preovulatory granulosa cells that had received an ovulatory dose of hCG (HEF + hCG) (2 h). Both protein-DNA complexes previously observed were present at all stages of follicular development (Fig. 3B, lanes 2–4). Additionally, similar protein-DNA complexes were formed with whole cell extracts isolated from R2C cells, a rat Leydig cell line (Fig. 3B, lane 5).

Role of the CAAT Box and E-box cis-Elements in trans-Activation of the PGS-2 Gene—When isolated from each other, the C/EBP (38) and E-box cis-elements appear capable of conferring functional activation of the PGS-2 promoter by binding specific factors. Therefore, it was essential to determine the requirement of each site within the context of an intact promoter. The mutations within the promoter were generated to disrupt the DNA elements binding C/EBPα and C/EBPβ (38), E-box proteins, or both types of transcription factors as discussed under “Experimental Procedures.” The three mutant
constructs, in addition to the wild type −195 PGS-2-CAT vector, were transiently transfected into differentiated granulosa cells as described previously (Fig. 4). The relative basal CAT activity in cells transfected with the −195 PGS-2 construct was similar to that obtained for the C/EBP Mt construct (11.5 ± 5 versus 12.3 ± 1.7, respectively). Likewise, the relative CAT activity (91 ± 26 versus 124 ± 47), as well as fold increase (10 ± 3 versus 10 ± 3-fold), induced by LH were similar for the −195 PGS-2-CAT and the C/EBP Mt, respectively. Thus, in the context of the intact promoter, the consensus CAAT box is not required for LH induction of PGS-2 promoter constructs in transient transfection assays of granulosa cells. However, when the E-box Mt was transfected into primary granulosa cell cultures, relative basal CAT activity in the absence of hormone was 15% of the activity produced by the −195 PGS-2-CAT construct. The relative CAT activity induced by LH was 15% of the activity of the nonmutated construct (−195 PGS-2-CAT). Since both basal and induced CAT activity decreased, the fold induction by LH was not affected (Fig. 4A). The Double Mt construct (harboring both the CAAT and E-box mutations) and the E-box Mt exhibited similar basal, as well as LH induced, relative CAT activity. The fold induction by LH decreased from 9- to 5-fold compared to the −195 PGS-2-CAT (Fig. 4A). These data provide further evidence that the E-box element is an essential DNA cis-element for the trans-activation of PGS-2 gene promoter constructs in differentiated granulosa cells.

Relative CAT activities induced by GnRH in cells transfected with the −195 PGS-2-CAT construct versus the C/EBP Mt were similar (40 ± 16 versus 50 ± 10; Fig. 4B). However, transfection of the E-box Mt decreased relative CAT activity induced by GnRH to 15% of the activity produced by the nonmutated construct (−195 PGS-2-CAT). The fold induction by GnRH remained unchanged for all three constructs. CAT activity in the presence or absence of GnRH in the Double Mt was similar and not different from that of the E-box Mt (Fig. 4B). These data
further demonstrate that the E-box is required for basal activity, as well as hormone induction, of PGS-2 transgenes in granulosa cell cultures.

CREB Does Not Bind the −67/−41 Region of the Rat PGS-2 Promoter—The mouse PGS-2 promoter −65/−39 bp oligonucleotide binds both CREB (36), c-Jun (37), and E-box proteins (36) in electrophoretic mobility shift assays. This same region is essential for v-src induction of PGS-2 chimeric genes in NIH 3T3 cells (37). To determine whether the protein-DNA complexes shifted by the rat −67/−41 oligonucleotide contained CREB, or other CRE-binding proteins, nuclear extracts of granulosa cells were incubated with an end-labeled rat −67/−41 oligonucleotide (Fig. 5A) in the presence or absence of unlabeled consensus CRE oligonucleotide or CREB antibody. The two complexes binding the rat E-box element were not competed by 100-fold excess of CRE; nor were they supershifted by 1:20 dilution of antibody to CREB. Neither the CRE nor CREB antibody altered the formation of the protein-DNA complexes I and II (arrows), C, when the mouse PGS-2 oligonucleotide was incubated with the same nuclear extract, complexes I and II as well as complexes III and IV were formed. Complexes III and IV, but not I and II, were decreased in the presence of the unlabeled CRE. Complex IV was supershifted (*) in the presence of CREB antibody (1:20). Thus, CREB and other protein CRE-binding proteins interact with the mouse, and not the rat, PGS-2 promoter.

E-box oligonucleotide, CREB was not present in rat or mouse complexes I and II (Fig. 5, B and C). By these in vitro DNA binding assays, it is clear that more proteins bind to this region of the mouse PGS-2 promoter than to the rat promoter, and that one of those proteins is CREB.

USF Binds to the E-box Region of the PGS-2 Promoter—Several class B E-box binding proteins have been identified and shown to bind the CACGTG consensus site (50). These include USF, which binds to this region when a thymidine (T) residue precedes the consensus region (TCACGTG) (50). To determine if USF was present in the E-box DNA-protein complexes, USF antibody was added to the binding reactions at increasing concentrations (dilutions of 1:2000, 1:200, and 1:20). Each dilution of the antibody generated a supershifted band, which was not observed in the presence of preimmune serum (Fig. 6A). The increasing amount of antibody in the binding reaction reduced the amount of protein/DNA present in both complexes I and II suggesting that both protein-DNA complexes contain the transcription factor USF.
This study has demonstrated that USF protein binds to the E-box in the rat PGS-2 promoter (Fig. 6A) and that this binding does not vary during follicular development (Fig. 3B). To determine if the USF protein is induced by LH in association with induction of the PGS-2 gene, differentiated granulosa cells were induced with hCG (500 ng/ml) for 2, 7, 12, and 24 h. Whole cell protein extracts collected prior to incubation with hCG (500 ng/ml) and after each time point were analyzed by immunoblot analysis. The amount of USF protein present in whole cell extracts of granulosa cells did not change after incubation with hCG (Fig. 6B). The doublet present on the immunoblot suggests that both the 43- and 44-kDa isoforms of USF are present in the granulosa cell lysates.

Promoters of Several Ovarian Genes Contain an E-box DNA cis-Element—Other genes which are involved in ovarian function contain a similar E-box consensus site in their 5'-flanking regions (Table I). The promoter of the RIIβ gene (regulatory subunit of protein kinase A) contains an E-box that binds both USF and c-myc present in Chinese hamster ovary (CHO) fibroblasts and mouse Nb2a neuroblastoma cell extracts (51). The gene encoding Ad4bp (adrenal 4 binding protein), also known as SF-1, requires an E-box for transcriptional activation of fusion constructs transfected into Y-1 adrenocortical cells and MA-10 testicular Leydig cells (52). Finally, the promoter of the FSH-receptor gene also has a consensus E-box (53) that is important for regulation of the FSH-receptor promoter fusion constructs in rat Sertoli cells (54).

To determine if the E-box elements present in the promoters bound similar proteins as the rat PGS-2 E-box, oligonucleotides corresponding to E-boxes of the human and rat PGS-2, as well as rat SF-1, rat FSH-R, and mouse RIIβ genes were synthesized, end-labeled and incubated with granulosa cell nuclear extracts. The labeled rat PGS-2 E-box oligonucleotide (~67/-41) formed two protein-DNA complexes (I and II) in the presence of nuclear extract as previously discussed (Fig. 7, panel A, lane 1). Complexes I and II were decreased in the presence of unlabeled competitor DNA corresponding to the rat PGS-2, RIIβ, and FSH-receptor E-box regions, but not to a CRE oligonucleotide (Fig. 7, panel A, lanes 2-5). In addition, both complexes were shifted in the presence of USF antibody (1: 2000; Fig. 7, panel A, lane 6).

The labeled human PGS-2 oligonucleotide formed several protein-DNA complexes when incubated with rat granulosa cell nuclear extract (Fig. 7, panel B, lane 7). These complexes were specifically competed by competitor human PGS-2 E-box DNA, but not with either rPGS-2 or RIIβ E-box competitor DNA (Fig. 7, panel B, lanes 8-10). Consensus CRE competitor DNA prevented formation of all, but two protein-DNA complexes (Fig. 7, panel B, lane 11). None of the complexes was shifted in the presence of the USF antibody (Fig. 7, panel B, lane 12).

TABLE I
Sequences of the oligonucleotides utilized in the electrophoretic mobility shift assays

| Promoter | E-box Oligonucleotide | Reference |
|----------|-----------------------|-----------|
| Rat PGS-2 | 5'-agg CAG TCA CGA AGT CAC GTG GAG TCC ACT | 31 |
| Mouse PGS-2 | 5'-agg TCA CGG GCA TGG CAC CTC AGG TGA gga | 36 |
| Human PGS-2 | 5'-agg TCA TGG CAC AGT GGC TCG GCA gga | 33 |
| SF-1 | 5'-agg TGG CAC AGT GGC TCG GCA GAG AA C GTC TCA GAC CCC CAC gga | 52 |
| Mouse RIIβ | 5'-agg ATG GAC GAC AGT GGC TCG GCA GAG | 51 |
| Rat FSH-R | 5'-agg G GTG GAT GCA GTG TAC CCG AAC CCA CAC CCA GGT GAC TGA AAC G gga | 53 |

Fig. 7. Promoters of several genes expressed in the ovary contain E-box elements that bind USF. Oligonucleotides containing E-box elements of several promoters were synthesized: A, rat PGS-2; B, human PGS-2; C, SF-1, D, RIIβ; and E, FSH receptor. The ability of each of these promoter sequences to bind proteins present in granulosa cell nuclear extracts was compared by EMSAs using labeled oligonucleotides as indicated in the presence of unlabeled competitor DNA (100-fold) or USF-antibody (1:2000) as indicated. See text for discussion.

rPGS-2 E-box competitor DNA (Fig. 7, panel C, lanes 1-4). These protein-DNA complexes were shifted in the presence of the USF antibody (Fig. 7, panel C, lane 5).

Labeled mouse RIIβ oligonucleotide formed two complexes...
similar to complexes I and II of the rPGS-2 E-box oligonucleotide (Fig. 7, panel D, lane 1). These complexes were decreased in the presence of RIIβ, FSH-R, and rPGS-2 competitor DNA, but not in the presence of a CRE oligonucleotide (Fig. 7, panel D, lanes 2–5). The complexes were shifted in the presence of a USF antibody (Fig. 7, panel D, lane 6). An additional protein-DNA complex is also present, but is not specifically competed by the unlabeled competitor DNA (Fig. 7, panel D, lanes 2–4).

Labeled FSH-receptor DNA formed two complexes similar to complexes I and II formed by the rat PGS-2, as well as the SF-1, E-box oligonucleotides (Fig. 7, panel E, lane 7). Formation of these complexes was prevented by competitor FSH-R, RIIβ, and rPGS-2 E-box DNA, but not the consensus CRE oligonucleotide (Fig. 7, panel E, lanes 8–11). In addition, both complex I and II were shifted in the presence of the USF antibody (Fig. 7, panel E, lane 12).

In summary, promoters for rat PGS-2, mouse PGS-2, RIIβ, FSH-R, and SF-1, but not human PGS-2 promoters, contain an E-box region capable of binding the USF protein present in granulosa cell nuclear extracts. The mouse PGS-2 and human PGS-2 promoters also contain a consensus CRE adjacent to the E-box, which binds the CREB protein (33, 36).

**DISCUSSION**

The specific molecular events controlling the expression of the PGS-2 gene in the ovary are dependent not only on the activation of specific signaling pathways, but also on the stage of follicular development and granulosa cell differentiation (55). Induction of PGS-2 mRNA and protein by protein kinase A (LH) and protein kinase C (GnRH) pathways occurs only in granulosa cells that have differentiated to a preovulatory phenotype (14, 24, 25, 26). This study documents that transcriptional regulation by LH and GnRH of rat PGS-2 promoter transgenes in rat ovarian granulosa cells is dependent on the integrity of a cis-acting E-box element located in the proximal promoter. Disruption of this E-box by a 5′ deletion to −52 bp, a point mutation that deletes the C nucleotide at position −52, or mutation of the consensus E-box sequence from CACGTG to CACCTT within the context of the −192 bp of the promoter caused marked decreases in the transcriptional activity of chimeric genes transfected into cultured rat granulosa cells. Mutations of a functional C/EBP consensus binding site (−140/−132) (38) within the context of the promoter did not abolish basal or hormonal regulation of the transgenes, indicating that the E-box region interacts with other cis-acting DNA elements located proximal to the CAAT box. Electrophoretic mobility shift assays documented that the transcription factor USF is present in granulosa cell nuclear extracts and binds to an E-box region of the rat PGS-2 promoter. No additional protein-DNA complexes were observed within this region indicating that USF is the primary transcription factor in granulosa cells that binds the E-box region of the rat PGS-2 promoter.

By comparing the binding activities of the rat, mouse, and human PGS-2 E-box regions using rat granulosa cell nuclear extracts, we have shown that the rat E-box region forms two complexes both of which contain USF. The E-box region of the mouse PGS-2 gene also binds USF, but in addition binds CREB and c-jun (36, 37). Despite the ability of the mouse PGS-2 promoter to bind each of these factors, c-jun is the transcription factor in NIH-3T3 cells that is able to mediate v-src transactivation of mouse PGS-2 transgenes in the absence of a functional E-box (37). A deletion mutant of the human PGS-2 promoter (−52/59) that disrupts the putative human E-box region also abolishes functional activity of chimeric transgenes. However, it is not yet known if the human PGS-2 E-box has functional importance for activity in any of the cell types tested or in conjunction with other regions (NF-κB, C/EBP, CRE) reported to confer functional activity (33, 34). In addition, it is not yet known which (if any) E-box proteins bind the putative E-box region of the human PGS-2 promoter. Although the human E-box (CACATG) differs from the rat and mouse (CACGTG) and does not bind USF, this sequence has been shown to bind other E-box proteins (50).

Although differences in PGS-2 promoter sequences between species may determine tissue-specific expression of the gene, the observed differences in the PGS-2 E-box regions of rat, mouse, and human do not appear to have major effects on the induction of PGS-2 in granulosa cells of preovulatory follicles. PGS-2 is induced by LH in rat (14, 24, 25), mouse (data not shown), bovine (56), and presumably human preovulatory granulosa cells. Furthermore, a mouse PGS-2 luciferase construct containing −371 bp of promoter sequence (36) is activated by LH when transfected into cultured rat granulosa cells (data not shown). Thus, the PGS-2 E-box of each species is likely to be functionally important in granulosa cells with the CRE/ATF binding sites providing additional regulation of activity in the mouse and human genes. Because the PGS-2 gene is usually repressed except in specific cell types and some transformed cell lines, there must also be factors that silence the promoter either by effecting the binding of USF or other regulatory factors.

Although USF was the only protein in granulosa cell extracts that bound to the rat PGS-2 E-box region in detectable amounts, it is unlikely to be the sole factor controlling PGS-2 expression in granulosa cells in response to agonist induction. USF protein levels and DNA binding activities were similar in small antral, preovulatory, and ovulatory granulosa cells exposed to hCG. This is not surprising since USF is ubiquitously expressed in all tissues (57). Therefore, changes in USF activity might be regulated by phosphorylation or by the interaction of USF with other components of the transcriptional machinery that are regulated or modified by the actions of LH and GnRH. Because no consensus phosphorylation sites have been found in the domains of the USF protein required for activation of transcription and because the putative USF protein activation domains are themselves not acidic activation domains, the mechanism of trans-activation by USF remains unclear (58). It appears likely that additional cis-acting factors and unknown co-activators may be involved in the hormonal regulation of the PGS-2 promoter in the rat ovary. Co-activators of USF have been identified in other tissues (59) and in the regulation of viral genes (60), however, the putative co-activator(s) of USF in ovarian cells remain to be determined.

The regulatory role of E-box regions is intriguing because several other genes expressed in ovarian granulosa cells have been shown to contain functional E-box sequences. These include genes that are developmentally regulated at an early stage of ovarian cell differentiation, such as SF-1 (52, 60–62) and the FSH receptor (63, 64), as well as genes that are hormonally regulated at later stages of granulosa cell differentiation, such as RIIβ (43, 65) and PGS-2 (15, 27, 38). As shown herein, the E-box of each of these genes binds USF present in rat granulosa extracts. The different patterns of expression of these genes during follicular development and in other selected tissues indicate that E-box regions exhibit different functional activities by interacting with different upstream regulatory regions and trans-activating factors in a cell specific manner. For example, although the E-box region of the mouse PGS-2 gene binds more proteins in fibroblasts than in granulosa cells, the E-box region of the mouse PGS-2 gene appears less important for the expression of PGS-2 transgenes in NIH 3T3 cells stimulated by v-src (36), than the E-box region of the rat PGS-2 genes expressed in rat granulosa cells stimulated by either LH.
or GnRH. Likewise, a region containing the E-box of the RIIβ gene is important for cAMP regulation in rat granulosa cells (43) and binds USF. Although the mouse RIIβ E-box binds more proteins in CHO extracts than in granulosa cell extracts, the magnitude of the effect of the E-box mutations in the RIIβ transgenes expressed in CHO and Nb2a cells (51) was far less than that observed for E-box mutants of SF-1 transgenes expressed in steroidogenic tissues (52) and E-box mutants of PGS-2 transgenes expressed in granulosa cells. Last, despite the presence of E-box proteins in CV-1 cells, SF-1 mRNA is not expressed in these cells; nor did these cells express SF-1 transgenes (52). Taken together, these observations indicate that the functional potency of the E-box regions of these genes is greater in steroidogenic cells than in fibroblasts and that the differences in potency likely depend on the relative amount and type of E-box binding proteins, as well as specific regions of the promoters and the cell specific factors binding to these regions. Thus, if USF is ubiquitous and constitutively expressed in granulosa cells, other factors must contribute to the developmental and hormone-induced expression of the PGS-2, RIIβ, SF-1, and FSH receptor genes at specific stages of granulosa cell differentiation by agonists.

The complexity of the regulation of the PGS-2 gene in multiple tissues is further underscored by the presence of other regions that confer activity in a cell specific manner. Previous studies in our laboratory (38) as well as those of others (33–35) have indicated that the consensus CAAT site binds C/EBPs and in certain conditions can confer agonist trans-activation of the PGS-2 gene. However, in the present study, when the CAAT site was mutated within the context of the intact promoter, no decrease in basal or agonist stimulated CAT activity was observed. Thus, although the CAAT box is capable of conferring agonist induction to the PGS-2 gene in the absence of the E-box cis-element, this may be due to the ability of C/EBPα and C/EBPβ to interact with the other components of the transcriptional machinery, whereas in the presence of USF, this interaction either does not occur, is not required or based on results with the human PGS-2 promoter, is altered to exert an inhibitory effect. Specifically, the activity of the human PGS-2 promoter in bovine arterial endothelial cells and of the mouse promoter in the osteoblastic cell line, MC3T3-E1, are highly responsive to the C/EBP site in an isoform specific manner (34, 35). C/EBPβ and not C/EBPα or C/EBPβ, is induced in bovine arterial endothelial cells by 12-O-tetradecanoylphorbol-13-acetate and lipopolysaccharide. Moreover, when a C/EBPβ expression vector was cotransfected with a human PGS-2 reporter construct into bovine arterial endothelial cells containing 327 bp of 5′-flanking sequence (including C/EBP, CRE, and putative E-box sequences), luciferase activity was markedly increased. Co-transfection with C/EBPβ and C/EBPα vectors decreased the response (34). In granulosa cells, C/EBPβ, rather than C/EBPα, mRNA, and protein are induced in response to LH, whereas C/EBPα is decreased (38). Therefore, C/EBPβ or C/EBPα may be candidates for factors that mediate the rapid decrease in trans-activation of the rat PGS-2 gene in response to the ovariatory LH surge.

In summary, transcriptional regulation of the PGS-2 gene is multifaceted. The trans-acting factors and cis-acting DNA elements that mediate trans-activation of the endogenous PGS-2 gene and chimeric PGS-2 transgenes appear to be dependent on the cell type, the agonists used to stimulate cellular signaling pathways and the species specific promoter elements. In this study we have shown that the integrity of the E-box region of the rat PGS-2 gene is required for expression in granulosa cells and appears to interact with upstream cis-acting elements other than the CAAT box to confer hormonal regulation of this gene in rat granulosa cells. The E-box region of the rat PGS-2 promoter binds USF (as do E-box regions of other genes regulated in the ovary), but does not contain ATF/CRE activity found in the human and mouse PGS-2 promoters.

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Academic Press, San Diego, CA

49. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1987) Current Protocols in Molecular Biology, pp. 12.2.1–12.2.3, John Wiley & Sons, New York

50. Bendall, A. J., and Molloy, P. L. (1994) Nucleic Acids Res. 22, 2801–2810

51. Singh, I. S., Luo, Z., Kozlowski, M. T., and Erlichman, J. (1994) Mol. Endocrinol. 8, 1163–1174

52. Nomura, M., Bärtsch, S., Nawata, H., Omura, T., and Morohashi, K. (1995) J. Biol. Chem. 270, 7453–7461

53. Heckert, L. L., Daley, I. J., and Griswold, M. D. (1992) Mol. Endocrinol. 6, 70–80

54. Goetz, T. L., and Griswold, M. D. (1995) Biol. Reprod. 50, Suppl. 1, 98

55. Richards, J. S. (1994) Endocr. Rev. 15, 725–751

56. Sirito, M., Lin, Q., Malty, T., and Sawadogo, M. (1994) Nucleic Acids Res. 22, 427–433

57. Kirschaum, B. J., Pognonec, P., and Roeder, R. G. (1992) Mol. Cell. Biol. 12, 5094–5101

58. Zawel, L., and Reinberg, D. (1993) Prog. Nucleic Acids Res. Mol. Biol. 44, 67–108

59. Richards, J. S. (1980) Physiol. Rev. 60, 51–89

60. Tilly, J. L., LaPolt, P. S., and Hsueh, A. J. (1992) Endocrinology 130, 1296–1302

61. Jahnsen, T., Hedin, L., Kidd, V. J., Beattie, W. G., Lohmann, S. M., Walter, U., Durica, J., Schulz, T. Z., Schiltz, E., Browner, M., Lawrence, C. B., Goldman, D., Ratoosh, S. L., and Richards, J. S. (1986) J. Biol. Chem. 261, 12352–12361