Molecular Characterization and Role of Bovine Upstream Stimulatory Factor 1 and 2 in the Regulation of the Prostaglandin G/H Synthase-2 Promoter in Granulosa Cells*

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The transcriptional activation of the prostaglandin G/H synthase-2 (PGHS-2) gene in granulosa cells is required for ovulation. To directly study the ability of upstream stimulatory factor 1 (USF1) and USF2 to trans-activate the bovine PGHS-2 promoter in granulosa cells, USF1 or USF2 expression vectors were co-transfected with the PGHS-2/luciferase (LUC) chimeric construct, −140–2PGHS-2-LUC. Results revealed that overexpression of USF1 or USF2 caused a marked and significant increase in basal and forskolin-inducible promoter activities (p < 0.05), and these effects were dependent on the presence of a consensus E-box cis-element within the promoter fragment. Co-transfections with different N- and C-terminal truncated USF mutants led to significant reductions in promoter activation, as compared with full-length constructs (p < 0.05), thus allowing identification of putative bovine USF functional domains. Overexpression of a USF2 truncated mutant lacking the first 220 residues (U2Δ1–220) acted as a dominant negative mutant and blocked endogenous and USF-stimulated PGHS-2 promoter activation. Interestingly, transfections with U2Δ1–220 blocked the forskolin-dependent induction of PGHS-2 mRNA in granulosa cells, whereas transfections with full-length USF2 increased PGHS-2 transcript levels. Immunoblot analyses confirmed overexpression of full-length and truncated USF proteins, and electrophoretic mobility shift assays (EMSA) and supershift EMSAs established that the observed effects were dependent on specific interactions between USF proteins and the consensus E-box cis-element. Stimulation of cells with forskolin increased, whereas treatment of extracts with phosphatase decreased USF binding activities to the E-box. Thus, this study presents for the first time direct evidence for a role of USF proteins in the regulation of the PGHS-2 promoter in preovulatory granulosa cells.

Prostaglandins are key mediators of inflammation, and the process of ovulation shares numerous signs of an acute inflammatory reaction, including hyperemia, edema, emigration of leukocytes, and induction of proteolytic and collagenolytic activities (1, 2). Likewise, there is a marked increase in intracellular levels of prostaglandin E₂ (PGE₂) and PGF₂α just prior to ovulation, and inhibitors of prostaglandin synthesis were shown to block ovulation in several species (3–6). Prostaglandin G/H synthase (PGHS, also known as COX) is the first rate-limiting enzyme in the biosynthesis of all prostaglandins from arachidonic acid, and two PGHS isoforms derived from distinct genes located on separate chromosomes have been characterized, and are referred to as PGHS-1 and PGHS-2 (7–9). A third PGHS isoform produced as an alternate splice variant of the PGHS-1 gene was recently identified as PGHS-3 (10). Several studies have established that the increase in follicular prostaglandin synthesis prior to ovulation is caused by a selective, gonadotropin-dependent induction of PGHS-2 (11–14). Within the preovulatory follicle, the enzyme was shown to be induced exclusively in granulosa cells, and not in theca interna (12, 14, 15). The obligatory role of PGHS-2 induction during ovulation was underscored in genetic studies in which ovulatory failure proved to be a common phenotype in PGHS-2-deficient female mice (16, 17).

The upstream stimulatory factor (USF) proteins belong to a family of transcription factors characterized by a C terminus containing basic region (B), helix-loop-helix (HLH), and leucine zipper (LZ) motifs responsible for dimerization and DNA binding activities (18, 19). Two distinct USF genes, referred to as USF1 and USF2, have been characterized and shown to encode full-length USF1 and USF2 proteins of 43 and 44 kDa, respectively (18–22). Alternative USF1 and USF2 splicing variants have also been identified, but their biological significance remains incomplete (22, 23). The C terminus B-HLH-LZ domains of USF1 and USF2 are highly conserved, and direct the formation of USF homodimers or heterodimers that interact with DNA with similar specificities (19, 20, 24). The cognate DNA binding cis-element for USF proteins, the E-box, contains the core nucleotide sequence CANNTG (18, 24). Another very well conserved domain between USF1 and USF2 is a small domain containing basic region (B), helix-loop-helix and leucine zipper (LZ) motifs.

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† The abbreviations used are: PGE₂, prostaglandin E₂; PG, prostaglandin; PGHS, prostaglandin G/H synthase; USF, upstream stimulatory factor; B-HLH-LZ, basic-helix-loop-helix-leucine zipper; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; TBE, 100 mM Tris, 100 mM borate, 2 mM EDTA; ANOVA, analysis of variance; ORF, open reading frame; FSK, forskolin; USR, USF-specific region; wt, wild type.

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tered USF-specific region (USR) that is located immediately upstream of the basic region and involved in nuclear localization and context-dependent transcriptional activation (25). In contrast, the N-terminal regions of USF1 and USF2 are much more divergent, but were shown to contain additional transactivation domains (25–27). USF proteins are ubiquitous transcription factors that play a key role during embryonic development (28) and that are known to activate or repress the expression of various genes (29–35).

The induction of PGHS-2 in granulosa cells prior to ovulation was shown to be dependent on transcriptional events (36). Initial studies on the regulation of the rat and bovine PGHS-2 promoter in granulosa cells revealed that the proximal 150–200 bp immediately upstream of the transcriptional start site were sufficient to confer basal and forskolin/gonadotropin inducible activities (37, 38). Different cis-elements were identified in this region, including a C/EBP, an ATF/CRE and an E-box element. However, site-directed mutagenesis studies showed only that the E-box played a central role in the regulation of the PGHS-2 promoter in granulosa cells (38–40). Endogenous USF proteins were detected in rat and bovine granulosa cells, and potential interactions with the E-box element were suggested from electrophoretic mobility shift assays (EMSA) (38–40). However, there has been no direct evidence demonstrating the capacity of USFs to trans-activate the PGHS-2 promoter in granulosa cells in any species. Therefore, the specific objectives of the present study were to clone and characterize the primary structure of bovine USF1 and USF2, and to provide direct evidence that USF proteins regulate PGHS-2 expression in preovulatory granulosa cells.

EXPERIMENTAL PROCEDURES

Materials—The QuickHyb hybridization solution and the ExAssist/ SOLR system were purchased from Stratagene Cloning Systems (La Jolla, CA). The bovine genomic library was obtained from Clontech (Bio/Can Scientific, Ontario, Canada). a-32PdCTP was purchased from PerkinElmer Canada Inc. (Woodbridge, Ontario, Canada). Lipo- fectAMINE PLUS reagent, TRizol total RNA isolation reagent, avian myeloblastosis virus-modified reverse transcriptase, 1-kb DNA ladder, synthetic oligonucleotides, pcDNA 3.1(+) was produced by subcloning into the multiple cloning site of the pBluescript vector (primers 18 and 19) and three antisense primers derived from bovine USF2 (primers 13, 20 and 21; Fig. 1, b and c). The first PCR amplification employed sense primer 18 and antisense primer 13, whereas the second and third PCR used primer pairs 19 and 20, and 19 and 21, respectively. The product of the third PCR was subcloned into pGEM-Teasy, sequenced and showed no miss only the first 18 bp of the USF2 open reading frame (ORF) (Fig. 1, c). The complete USF2 coding region was isolated by RT-PCR using 0.5 μg of preovulatory follicle RNA and sense primer 7 (designed from the highly conserved 5′-end of human and mouse USF2 ORF) and antisense primer 8 (Fig. 1, d).

Bovine USF1 and USF2 cDNA and PGHS-2 Promoter Constructs—Expression constructs containing full-length USFs, referred to as wild type USF1 (U1wt) and USF2 (U2wt), were produced by subcloning into pcDNA 3.1(+) (Invitrogen). Constructs expressing N terminus truncated forms of USF1 were produced by PCR using U1wt as template and sense primers 3, 4, or 5 and common antisense primer 2 to generate mutants lacking residues 1–57 (U1L1–57), 1–129 (U1L1–129), or 1–186 (U1L1–186), whereas a C terminal-truncated USF1 lacking residues 249–310 (U1249–310) was produced with sense primer 1 and anti- sense primer 6 (Fig. 1a, c). Constructs expressing N terminus truncated forms of USF2 were produced by PCR using U2wt as template and sense primers 9, 10, or 11 and common antisense primer 8 to generate mutants lacking residues 1–149 (U2L1–149), 1–182 (U2L1–182), or 1–220 (U221–220), whereas a C terminal-truncated USF2 lacking residues 299–346 (U2349–346) was produced with sense primer 7 and antisense primer 12 (Fig. 1b, e). All constructs were sequenced to confirm their identity.

The production of bovine PGHS-2 promoter-firefly luciferase chimera constructs, −149/−2PGHS-2-LUC and −149/−2PGHS-2ΔE-box-LUC, has been previously described (38). The −149/−2PGHS-2-LUC was produced by subcloning the PGHS-2 promoter fragment −149/−2 into the multiple cloning site of the pBluescript vector (Promega), whereas the −149/−2PGHS-2ΔE-box-LUC was designed to contain a mutated E-box element in the context of the −149/−2PGHS-2 promoter frag- ment (38).

Cell Culture, Transient Transfections, and Reporter Activity Assays—Primary cultures of bovine granulosa cells, transient transfections, and reporter activity assays were performed as previously described (38). Briefly, cells were seeded in 24-well plates at a density of 1–2 × 103 cells/0.5 ml of minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2% fetal bovine serum, 100 U/ml penicillin (1 μg/ml), transferrin (5 μg/ml), and penicillin (100 units/ml)/streptomycin (100 μg/ml), and incubated at 37 °C in a humidified atmosphere of 5% CO2. Cultures were transfected transiently with 90 fmol/well of −149/−2PGHS-2-LUC or −149/−2PGHS-2ΔE-box-LUC in the absence or presence of various USF expression constructs (1–50 fmol/well) using 2 μg of LipofectAMINE PLUS reagent, following the manufacturer’s protocol. Co-transfection with the SV40 Renilla luciferase control vector (pRLSV40; Promega) was performed to normalize results. Three hours after transfection, cells were incubated in fresh culture media for 36 h in the absence or presence of forskolin (10 μM). At the end of the culture period, cell lysates were collected and firefly and Renilla luciferase activities were determined using Promega’s Dual Luciferase Assay System and a Lumat LB 9507 luminometer (Berthold Technologies).

Cell Extracts and Immunoblot Analysis—Cell extracts were prepared as described (12), and protein concentrations were determined by the
method of Bradford (43) (Bio-Rad Protein Assay). Samples (25–50 μg of proteins) were resolved by one-dimensional SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes (38, 44). Membranes were incubated with anti-USF1 (1:200), USF2 (1:800) or PGHS-2 antibody (1:7500), and immunoreactive proteins were visualized by incubation with the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:3000 dilution) and the enhanced chemiluminescence detection system (ECL plus) according to the manufacturer's protocol (Amersham Biosciences).

**Granulosa Cell Nuclear Extracts and Electrophoretic Mobility Shift Assays (EMSA) —** Bovine granulosa cells were cultured in the presence or absence of forskolin (10 μM) for 36 h, and nuclear extracts were prepared as described (37, 38). Protein concentration in each extract was determined by the method of Bradford (43). EMSAs were performed as described (37, 38), with minor modifications. Briefly, extracts of nuclear proteins (0.5 μg/reaction) were incubated with 40,000 cpm of end-labeled 149/149’-2 PGHS-2 promoter fragment and 1 μg of poly(dI/dC) (Amersham Biosciences) in a final volume of 20 μl of binding buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM KCl, 5 mM MgCl₂, 5 mM dithiothreitol, and 12% (v/v) glycerol. In some experiments aimed at studying the effect of protein dephosphorylation on binding activity, extracts were preincubated for 10 min with phosphatase type VII-5 (0.5–1 IU; Sigma) prior to the addition of other reagents. When antibodies were used in supershift EMSAs, the nuclear extract was first incubated for 20 min with the antisera prior to the addition of other reagents. Binding complexes were resolved by 5% acrylamide, 0.5 mM Tris borate/EDTA gel electrophoresis.

**RNA Extraction and RT-PCR Analysis —** Total RNA was extracted from granulosa cells cultured under various experimental conditions using TRIzol (Invitrogen). The Access RT-PCR System (Promega) was used to characterize expression of PGHS-2 and GAPDH transcripts. Reactions were performed with sense and antisense primers specific for PGHS-2 (5’-CACAGTGCACTACATACTACACC-3’ and 5’-TCTCTGCACACAAGAGCTCATC-5’) or PGHS-2 antibody (1:7500), and immunoreactive proteins were visualized by incubation with the horseradish peroxidase-linked donkey anti-rabbit secondary antibody (1:3000 dilution) and the enhanced chemiluminescence detection system (ECL plus) according to the manufacturer’s protocol (Amersham Biosciences).

**Statistical Analyses —** One-way ANOVA was used to test the effect of different treatments and constructs on reporter gene activity. When
The translation initiation (ATG) and stop (TAA or TGA) codons are shown in amino acid sequences of the bovine USF2 cDNA. The cDNA is composed of an ORF of 1038 bp (uppercase letters) an da3/H11032sequences were deposited to GenBank™ (accession numbers AY241931 and AY239291, respectively).

The bovine USF2 cDNA was composed of an ORF of 1041 bp (uppercase letters) and a 3'-untranslated region of 268 bp (lowercase letters). The translation initiation (ATG) and stop (TAA or TGA) codons are shown in amino acid sequences of the bovine USF1 cDNA. The cDNA is composed of an ORF of 933 bp (uppercase letters) an da3/H11032sequences were deposited to GenBank™ (accession numbers AY241931 and AY239291, respectively).

ANOVAs indicated significant differences (p < 0.05), Dunnett’s test was used for multiple comparisons of means. Statistical analyses were performed using JMP software (SAS Institute, Inc., Carry, NC).

RESULTS

Characterization of Bovine USF1 and USF2—The isolated bovine USF1 cDNA was composed of an ORF of 933 bp (including the stop codon) and a 3'-untranslated region of 301 bp (Fig. 2A). Its coding region encodes a 310-amino acid protein, which is identical in length to other known mammalian USF1 proteins (18, 45–47). Overall comparisons between bovine USF1 and the human (18), mouse (45), rat (46), and rabbit (47) homolog revealed a 97% identity at the amino acid level. The bovine USF2 cDNA was composed of an ORF of 1041 bp encoding a 346-amino acid protein, and a 3'-untranslated region of 268 bp (Fig. 2B). Comparisons between bovine USF2 and the human (19), mouse (20), and rat (48) homolog revealed a 97% identity at the amino acid level.

USFs- and E-box-Dependent Transactivation of the Bovine PGHS-2 Promoter in Granulosa Cells—To provide direct evidence that USF proteins transactivate the bovine PGHS-2 promoter in granulosa cells, USF1 or USF2 expression vectors were co-transfected with the −149/−2PGHS-2.LUC construct, which contains a mutated E-box element in the context of the −149/−2PGHS-2 promoter fragment. Results showed that transfections with USF1 and USF2 produced levels of luciferase activity similar to those observed with USF2 alone (data not shown). To investigate the role of the E-box element in PGHS-2 promoter activities, transfection experiments were performed with the −149/−2PGHS-2.LUC construct, which contains a mutated E-box element at −149 in the bovine PGHS-2 promoter. Basal and forskolin-dependent activities were also ablated when granulosa cells were cotransfected with the mutated promoter construct and with USF1 or USF2 (Fig. 3, A and C). Cotransfections with USF1 and USF2 had a marked effect on promoter activities, with a 3.3- and 6.6-fold increase in basal and forskolin-inducible activities, respectively (Fig. 3A). Likewise, a 5.2- and 8.6-fold increase in basal and inducible activities, respectively, were observed after cotransfections with USF2 (Fig. 3A), clearly establishing the capacity of USF proteins to activate the PGHS-2 promoter in granulosa cells. The stimulatory effect of each USF construct was dose-dependent, with the highest amount (50 fmol) of USF1 or USF2 causing a 6.7- and 14-fold increase in basal activity, and 21.9- and 38.4-fold increase in forskolin-inducible activities, respectively (Fig. 3A). Cotransfections with USF1 and USF2 produced levels of luciferase activity similar to those observed with USF2 alone (data not shown).

To investigate the role of the E-box element in PGHS-2 promoter activities, three truncated mutants were generated and co-transfected with the −149/−2PGHS-2.LUC construct, which contains a mutated E-box element at −149 in the bovine PGHS-2 promoter. Basal and forskolin-dependent activities were also ablated when granulosa cells were cotransfected with the mutated promoter construct and with USF1 or USF2 (data not shown).

Truncated USFs Repress PGHS-2 Promoter Activation—To delineate potential functional domains in bovine USF proteins, several truncated mutants were generated and co-transfected with the −149/−2PGHS-2.LUC promoter construct in granulosa cells. Studies with USF1 mutants revealed that deletion of
Results are presented as relative luciferase activity (firefly/Renilla) or presence of wild type USF1 (U1wt) and USF2 (U2wt) expression vectors, respectively, as compared with full-length USF1 wildtype (U1wt) (p < 0.05; Fig. 4B). In contrast, a marked reduction in basal activity was observed for all three constructs (p < 0.05; Fig. 4B). The deletion of 48 residues at the C terminus (U2Δ299–346) completely abolished the ability of the protein to stimulate promoter activities above those observed with −149/−2PGHS-2.LUC alone (Fig. 4B). Overexpression of full-length U2wt and truncated U2Δ1–220 was confirmed by immunoblot (Fig. 4D).

USF1 and USF2 can interact with DNA as homodimers and heterodimers through their basic helix-loop-helix (bHLH) and leucine zipper (LZ) domains (19, 20, 24). To characterize the ability of U1Δ1–129 or U2Δ1–220 to dimerize with full-length U1wt and U2wt, and affect PGHS-2 activation, each mutant was co-transfected with wild type USFs and −149/−2PGHS-2.LUC in granulosa cells. Results showed that U1Δ1–129 did not reduce U1wt-stimulated basal activity, reduced by only 35% forskolin-inducible U1wt-driven activity, and did not significantly alter U2wt-driven PGHS-2 promoter activities (Fig. 5A). In contrast, U2Δ1–220 decreased by 38 and 64% basal and inducible activities stimulated by U1wt, and reduced by 62 and 73% basal and inducible activities stimulated by U2wt, respectively (Fig. 5A), clearly suggesting the ability of U2Δ1–220 to dimerize with full-length USFs and decrease PGHS-2 activation. Interestingly, under present experimental conditions (i.e. simultaneous co-transfection with U2Δ1–220 and −149/−2PGHS-2.LUC), U2Δ1–220 was unable to suppress basal and forskolin-inducible activities to levels below those obtained with the −149/−2PGHS-2.LUC alone (Fig. 5A). However, when cells were first transfected with U2Δ1–220 and cultured for 24 h to allow expression of truncated USF2 prior to a second transfection with −149/−2PGHS-2.LUC, U2Δ1–220 was able to block endogenous basal and inducible activities by 77 and 78%, respectively (p < 0.05; Fig. 5B). Likewise, pre-expression of U2Δ1–220 for 24 h exerted a strong inhibitory action on U2wt-stimulated promoter activities (p < 0.05; Fig. 5B).

To determine whether the inhibitory effect of U2Δ1–220 on PGHS-2 promoter activation could translate into the regulation of PGHS-2 transcripts in granulosa cells, cells were transfected with U2Δ1–220 24 h prior to forskolin stimulation, and RT-PCR was performed to study changes in mRNA levels. Results showed that U2Δ1–220 blocked the forskolin-dependent induction of PGHS-2 mRNA in granulosa cells (Fig. 5C), whereas transfections with the empty expression vector had no effect (data not shown). In contrast, cells transfected with full-length U2wt led to higher basal and forskolin-dependent induction of PGHS-2 transcripts (Fig. 5C), in keeping with effects observed on promoter activities (Fig. 5A).

**Binding Activity of USF Proteins to the E-box cis-Element within the PGHS-2 Promoter**—To relate the ability of USF proteins to trans-activate the PGHS-2 promoter to specific protein-DNA interactions, EMSAs were performed with nuclear extracts prepared from granulosa cells transfected with USF constructs and the −149/−2 PGHS-2 promoter fragment. Results showed that a major protein/DNA complex was formed with cells transfected with U1wt or U2wt (Fig. 6A, lanes 3 and 4, respectively), with the U1wt band migrating slightly faster than U2wt in keeping with minor differences in their molecular.

**Fig. 3.** USFs- and E-box-dependent regulation of the bovine PGHS-2 promoter in granulosa cells. A, bovine granulosa cells were co-transfected with promoterless plasmid pGL3.Basic (Basic), −149/−2PGHS-2.LUC (−149/−2PGHS) or the E-box-mutated construct −149/−2PGHS-2ΔE-box.LUC (−149/−2PGHSΔE-box) in the absence (−) or presence of wild type USF1 (U1wt) and USF2 (U2wt) expression constructs, as described under “Experimental Procedures.” All cultures were also co-transfected with the SV40 Renilla luciferase vector (pRL.SV40) as an internal control to normalize experimental reporter activity. After transfection, cells were cultured for 36 h in the absence (control) and presence of forskolin (10 μM; FSK). B, co-transfection with −149/−2PGHS and graded doses (0–50 fmol/well) of U1wt. C, co-transfection with −149/−2PGHS and graded doses (0–50 fmol/well) of U2wt. Results are presented as relative luciferase activity (firefly/Renilla; mean ± S.E. of triplicate cultures from four experiments).

the first 129 amino acid residues (U1Δ1–129) had the strongest effect on PGHS-2 promoter activities, leading to a 63 and 91% reduction in basal and forskolin-inducible activities, respectively, as compared with full-length USF1 wildtype (U1wt) (p < 0.05; Fig. 4A). Other N-terminal (U1Δ1–57, U1Δ1–186) and C-terminal (U1Δ249–310) truncated USF1 constructs also led to marked reductions in promoter activities, as compared with U1wt (p < 0.05; Fig. 4A). The apparent increase in promoter activities between the U1Δ1–129 and the U1Δ1–186 construct was not expected, and the precise reasons for this observation remain unclear. Immunoblot analyses confirmed the overexpression of full-length U1wt and truncated U1Δ1–129 protein in transfected granulosa cells (Fig. 4C). Studies with USF2 mutants showed that deletion of the first 149 (U2Δ1–149), 182 (U2Δ1–182), and 220 (U2Δ1–220) amino acid residues led to progressive reduction in forskolin-inducible activity, as compared with full-length USF2 wild type (U2wt), with U2Δ1–220 producing only 7% of that of U2wt (p < 0.05; Fig. 4B).
weight. A larger complex encompassing both USF bands was observed when cells were co-transfected with U1wt and U2wt (Fig. 6A, lane 5). Stimulation with forskolin did not change the nature of the bands, but an apparent increase in the intensity of the complexes was observed (compare lanes 7, 8, and 9 with lanes 3, 4, and 5, respectively; Fig. 6A).

To characterize the specificity of protein/DNA interactions, and to establish the role of the E-box element present in the −149/−2 PGHS-2 promoter fragment, EMSAs were performed using molar excess of various competitor DNA. Results showed that U1wt and U2wt complexes were competed with excess unlabeled wild type −149/−2 (lane 2 versus 1, and lane 6 versus 5; Fig. 6B) or excess complementary oligonucleotides containing the bovine E-box element (lane 4 versus 1 and lane 8 versus 5, Fig. 6B), but were not competed with cold −149/−2 DNA fragment in which only the E-box was mutated (lane 3 versus 1 and lane 7 versus 5; Fig. 6B). In a complementary experiment, no protein/DNA complex was observed when the same extracts were incubated with labeled −149/−2PGHS-2 DNA fragment containing a mutated E-box element (lanes 2 and 3, Fig. 6C). Collectively, these results illustrate the central role of the E-box in producing USFs/DNA binding activities.

To confirm the presence of USF proteins in binding complexes, supershift EMSAs were performed using antibodies against USF1 and USF2. The major band produced with extracts prepared from cells transfected with U1wt was markedly shifted with the anti-USF1 antibody but was not affected by the anti-USF2 antibody (lanes 1, 2, and 3, Fig. 7). Conversely, the band produced with the U2wt extract was shifted with anti-USF2, but not with anti-USF1 antibody (lanes 7, 8, and 9; Fig. 7). Interestingly, extracts prepared from cells transfected with N-terminal truncated U1Δ1–129 and U2Δ1–220 generated bands with higher mobility that were also shifted by their respective antibody (lanes 4 and 5 and lanes 10 and 11, Fig. 7). In contrast, no complex was formed with extracts from cells expressing C-terminal truncated U1Δ249–310 and U2Δ299–346, which lack portion of the HLH and all the LZ domain (lanes 6 and 12, Fig. 7).

Phosphatase Treatment Decreases USFs/DNA Binding Activities—To study the effect of protein dephosphorylation on USF1 and USF2/DNA binding activity, nuclear extracts from cells transfected with U1wt or U2wt cultured in the absence or presence of forskolin were treated with phosphatase prior to EMSAs. Results showed that phosphatase treatment decreased
the intensity of U1wt and U2wt bands (Fig. 8). However, binding complexes produced with extracts from forskolin-treated cells appeared more resistant to phosphatase treatment than extracts from untreated cells, especially with the U2wt cell extract (lanes 4–6 versus lanes 1–3, Fig. 8B).

**DISCUSSION**

The process of follicular rupture is essential to all mammalian reproductive cycles, and numerous investigations have established that the induction of prostaglandin synthesis is required for ovulation (1–4, 16, 17). A number of studies revealed that the obligatory preovulatory rise in follicular prostaglandin synthesis results from a selective, gonadotropin-dependent induction of PGHS-2 transcript and protein in granulosa cells (11–14). The previous characterization of the rat and bovine PGHS-2 promoter showed that a conserved E-box element located in the proximal promoter region plays a central role in basal and agonist inducible activities in granulosa cells (37, 38). The present study provides for the first time direct evidence that USF proteins *trans*-activate the PGHS-2 promoter in granulosa cells. Using the bovine model, our results demonstrate that overexpression of full-length USF1 and USF2 proteins resulted in a marked increase in PGHS-2 mRNA in granulosa cells. These effects involved specific interactions between USF proteins and the consensus E-box element located between −149 and −50 bp upstream the bovine PGHS-2 transcription start site.

The present study also establishes some of the functional domains of bovine USF proteins. Deletion of USF1 and USF2 regions upstream of the USR-B-HLH-LZ domain resulted in a marked decrease in PGHS-2 promoter activation but had no effect on DNA binding activity, in keeping with the presence of
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Pro\textsuperscript{220} appeared central for forskolin-inducible promoter activation. The other extremity of the bovine USF proteins was equally important for their overall activity, as removal of the last 62 and 48 amino acid residues of USF1 (U1\textDelta249–310) and USF2 (U2\textDelta299–346), respectively, resulted in a loss of their capacity to stimulate transcription. However, in these latter cases, the inability of C-terminal-truncated USF mutants to bind to the E-box element present in the proximal PGHS-2 promoter fragment appeared as the underlying mechanism responsible for the loss of activity, in keeping with the dimerization/DNA binding functions of the HLH-LZ domains identified in this region (18–20, 24).

The production of a bovine USF dominant negative mutant was sought as an important tool to demonstrate the functional role of the transcription factor in the regulation of the PGHS-2 promoter in granulosa cells. This approach has been used previously to establish the function of USF proteins in the regulation of other promoters (49–53). However, although N-terminal and C-terminal truncations of USF1 and USF2 reduced or blocked their trans-activating capacity, these mutants were unable to inhibit endogenous PGHS-2 promoter activities when transfected simultaneously with the promoter construct. As a potential explanation for this outcome, it was hypothesized that a rapid stimulation of basal and forskolin-inducible PGHS-2 promoter activities by endogenous USF proteins preceded expression of sufficient USF mutants when both constructs were co-transfected contemporaneously. The hypothesis appeared supported by results from experiments in which endogenous activities were abrogated when cells were allowed to accumulate a USF mutant (U2\textDelta1–220) for 24 h prior to transfection with the PGHS-2 promoter construct. EMSAs and supershift EMSAs confirmed the ability of overexpressed U2\textDelta1–220 to bind the E-box element present in the bovine PGHS-2 promoter, thus establishing the saturation of a critical cis-element by non-functional truncated transcription factors as the putative mechanism of action for this dominant negative mutant.

While this study establishes that binding of USF proteins to the E-box is a prerequisite for the regulation of the PGHS-2 promoter in granulosa cells, the complete molecular control of PGHS-2 induction during ovulation remains to be characterized. Importantly, previous studies have shown that USF proteins are expressed at constant levels in rat and bovine granulosa cells during the ovulatory process, indicating that PGHS-2 induction is not caused by changes in levels of these transcription factors (38, 39). Alternatively, the involvement of coactivator(s) regulated by the LH preovulatory surge may control the transcriptional activity of USFs in granulosa cells. The presence of a specialized unidentified coactivator has been implicated in the transcriptional activity of USF proteins in HeLa cells (54). The coactivator p300, which has intrinsic acetyltransferase activity, was shown to interact with USF2 to potentiate transactivation of the mouse cytochrome c oxidase subunit Vb promoter (51) and the human telomerase reverse transcriptase promoter (55), and was thus considered as a potential candidate for the PGHS-2 promoter in granulosa cells. However, co-transfections with a p300-expressing construct had no effect on USF1 or USF2 regulated PGHS-2 promoter activity in granulosa cells (data not shown). This result is in keeping with another report that revealed that p300 was unable to affect USF1-dependent transactivation of the TGF-\beta2 promoter in F9 differentiated embryonal carcinoma cells (56), suggesting that the involvement of p300 in USF action may be promoter- and cell-specific.

The preovulatory surge of luteinizing hormone (LH) is the primary physiological trigger of follicular PGHS-2 induction
and ovulation (1, 2). The LH receptor (LHR) is a member of the rhodopsin/7-transmembrane receptor subfamily of G protein-coupled receptors, and LHR-mediated effects in preovulatory granulosa cells are directed mostly, but not exclusively, through the activation of the G protein/adenyl cyclase/cAMP/cAMP-dependent protein kinase (PKA) pathway (57). The phosphorylation of transcription factors by activated PKA represents an important mechanism by which gene expression is controlled (58). The stimulatory effect of forskolin, a potent activator of adenylyl cyclase, on endogenous and USF-driven PGHS-2 promoter activities in the present study raises the possibility that USF phosphorylation may be involved in the regulation of the promoter in granulosa cells. A number of reports have documented that USF1 can be phosphorylated by various kinases, including cyclin A2 and B1-p34CDC2, stress-responsive p38 kinase, PKA and proteine kinase C, and that USF1 phosphorylation led to increases in DNA binding and transactivation of different promoters (59–61). Interestingly, results from the present study showing apparent forskolin-dependent increase, and phosphatase-dependent decrease, in USFs binding activities to the E-box also argue for a potential role of USF phosphorylation in PGHS-2 transcriptional regulation.

Lastly, this study reports for the first time the cloning and characterization of bovine USF1 and USF2, with results underscoring the remarkable similarities among the primary structure of USF1 across species. The length of bovine USF1, 310 amino acids, is identical to that of the four other mammalian orthologs characterized thus far (18, 45–47). More importantly, comparative analyses revealed that the amino acid sequence of bovine USF1 is almost identical to that of other species, being 99% identical to human (18) and 97% identical to mouse (45), rat (46), and rabbit USF1 (47). Comparisons at the nucleic acid level also revealed that the corresponding coding region of the bovine USF1 cDNA was highly conserved, being 89–94% identical to these mammalian orthologs (18, 45–47). As for bovine USF2, the size of the full-length protein (346 amino acids) is identical to that of the three other characterized mammalian counterparts (19, 20, 48), and levels of similarities among amino acid sequences (97% identity) and corresponding nucleic acid sequences (90–92% identity) were very high when the bovine factor was compared with human (19), mouse (20), and rat USF2 (48). Consequently, all putative structural and functional domains are thought to be conserved in bovine USF proteins, including the characteristic C terminus B-ILH-LZ domains responsible for dimerization and DNA binding activities (18, 19, 24). It should also be noted that, as for USF2 of other species, the bovine USF2 N terminus was particularly rich in guanine and cytosine residues. The high GC content of the first 150 nucleotides (72% GC) and 400 nucleotides (69% GC) located immediately after the translation initiation codon were likely responsible for difficulties encountered in cloning the 5-end of bovine USF2.

In summary, this is the first study to characterize the primary structure of bovine USF1 and USF2, and to provide direct evidence that USF proteins are involved in the trans-activation of the PGHS-2 promoter in granulosa cells. Thus, considering the obligatory role of the gonadotropin-dependent induction of PGHS-2 in ovarian follicles during the ovulatory process, this study clearly contributes to our understanding of the molecular mechanisms responsible for this key physiological process. However, more efforts will be needed to unravel the precise molecular control of USF action on PGHS-2 induction in granulosa cells, with future studies focusing on the potential role of USF phosphorylation and involvement of transcriptional coactivators.
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Molecular Characterization and Role of Bovine Upstream Stimulatory Factor 1 and 2 in the Regulation of the Prostaglandin G/H Synthase-2 Promoter in Granulosa Cells

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