Prostaglandin endoperoxide synthase isoform 2 (PGS-2) mRNA and protein are transiently induced by gonadotropins in granulosa cells of preovulatory follicles prior to ovulation. To better understand the hormonal regulation of the rat PGS-2 (rPGS-2) gene in these cells, genomic clones containing rPGS-2 as well as up to 6 kilobases of 5′-flanking DNA were isolated by screening a rat liver genomic library with a labeled 5′-fragment of the mouse PGS-2 cDNA. Primer extension analysis using ovarian follicular mRNA identified the 3′-end of a single rPGS-2 transcription initiation site located 44 nucleotides upstream of the ATG translation initiation codon. To test for promoter activity within the 5′-flanking region of the rPGS-2 gene, a genomic fragment, −2698/321 (1 =cap site), as well as a series of 5′-deletion mutants, were fused upstream of the chloramphenicol acetyltransferase (CAT) reporter gene and transfected into primary cultures of granulosa cells. Forskolin (7.5 μM), follicle-stimulating hormone (500 ng/ml) and luteinizing hormone (500 ng/ml) induced CAT activity following transfection with the −2698/32PGS*CAT, whereas gonadotropin-releasing hormone (100 nM) and interleukin-1β (30 ng/ml) had no effect. Deletion mutants delineated the region spanning from −192 to −54 of the transcription start site to be essential for both basal and forskolin-regulated expression of the reporter gene. The same DNA fragment (−192/−54) exhibited specific binding to granulosa cell nuclear extract proteins as analyzed by electrophoretic mobility shift assays. Additional specific bands were observed in extracts prepared from granulosa cells exposed to an ovulatory dose of gonadotropins in granulosa cells involves 5′-flanking DNA sequences, specifically a region between −192 and −54 of the transcription initiation site.

Characterization and Hormonal Regulation of the Promoter of the Rat Prostaglandin Endoperoxide Synthase 2 Gene in Granulosa Cells

IDENTIFICATION OF FUNCTIONAL AND PROTEIN-BINDING REGIONS*

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Prostaglandin endoperoxide H synthase (PGS) is the first rate-limiting enzyme in the biosynthetic pathway of prostaglandins from arachidonic acid (for recent reviews see Refs. 1-3). The native synthase is a homodimer composed of two subunits and exhibits both cyclooxygenase and peroxidase activities. Results from recent studies have clearly established the presence of two distinct PGS enzymes. The first characterized PGS isoform, now referred to as PGS-1, was purified more than 15 years ago from ovine and bovine seminal vesicles (oPGS-1, 4, 5; hPGS-1, 6). The cDNA for oPGS-1 was cloned from an ovine seminal vesicle expression library and shown to encode a 2.8-kilobase RNA transcript (7-9). The oPGS-1 cDNA was subsequently used to isolate cDNAs encoding murine (mPGS-1, 10) and human PGS-1 enzymes (hPGS-1, 11). The amino acid sequences of PGS-1, as deduced from the respective cDNA of each species, were shown to be highly (90%) similar (7-11). The genes for hPGS-1 and mPGS-1 genes have also been characterized, each being comprised of 11 exons and 10 introns and approximately 22 kilobases in length (12, 13).

More recently, a second isoform of PGS, referred to as PGS-2, has been identified in chicken (14, 15) and mouse fibroblasts (16-18), in rat granulosa cells (19, 20), and in human endothelial cells (21). Primary amino-termina amino acid sequence of rat ovarian PGS-2 (19), as well as deduced mouse and cow, and human PGS-2 nucleotide regions, were shown to be approximately 60% similar to PGS-1. Although important structural and putative functional domains of the PGS-1 enzyme are highly conserved in PGS-2 (i.e. N-linked glycosylation sites, a hydrophobic transmembrane domain, the active-site tyrosine, proximal and distal heme-binding domains, and the aspirin acetylation site), the PGS-2 enzyme is clearly derived from a distinct gene that consists of 10 exons and 9 introns, and is about 8 kilobases in length (17, 22). Lastly, mRNA transcripts of the PGS-2 gene are approximately 4.0-4.5 kilobases (15, 16, 18, 20, 21, 23, 24) compared to 2.8 kb for PGS-1 (7-9).

Not only are there two distinct genes encoding the two PGS isoforms, but the regulated expression of each gene exhibits a high degree of agonist and tissue specificity. For example,
PGS-1 appears to be constitutively expressed in several tissues including ovine seminal vesicle (4, 5), mouse fibroblasts (16, 18), rat kidney and uterus (19, 25), thymus cells of ovarian follicles (25), and alveolar macrophages (24). In contrast, PGS-2 mRNA is induced by multiple agonists in immortal mouse fibroblasts (16, 18), by permissive temperature in chicken embryo fibroblasts transformed by the Rous sarcoma virus (14), by bacterial lipopolysaccharide in alveolar macrophages (24), and by gonadotropins in granulosa cells of preovulatory ovarian follicles prior to ovulation (19). Furthermore, gonadotropin induction in vivo of PGS-2 mRNA and protein in granulosa cells of preovulatory follicles can be mimicked by two models in vitro using gonadotropins and forskolin, as well as the decapeptide GnRH (20, 25). In all cases, induction of PGS-2 is rapid but transient.

To understand the molecular mechanisms regulating the differential expression of the PGS genes, 5'-flanking regions of the mouse PGS-1 (13) and PGS-2 (17) genes have been cloned, sequenced, and the transcription initiation site characterized. Although different putative response elements (AP-1 and Sp1) were identified in these 5'-flanking DNA sequences, their functional relevance remains to be determined. The general objective of the present study was to clone and characterize the promoter of the rat PGS-2 gene in order to analyze functional domains involved in transcriptional activation of rPGS-2 in granulosa cells by gonadotropins. For this, 5'-flanking sequences of the putative promoter and a series of 5'-deletion mutants were fused upstream of the chloramphenicol acetyltransferase (CAT) reporter gene. These constructs were assayed by transiently transfecting primary cultures of granulosa cells exhibiting a preovulatory phenotype and in which luteinizing hormone induces PGS-2 mRNA (20). Lastly, studies were performed to determine if proteins present in nuclear extracts of granulosa cells prior to and after exposure to an ovulatory dose of human chorionic gonadotropin (hCG) were able to bind to the DNA region involved in the activation of the rat PGS-2 promoter.

EXPERIMENTAL PROCEDURES

Materials—Immature female rats (24-26 days of age) and adult pregnant rats were purchased from Holtzman (Madison, WI); hypophysectomized immature female rats (day 26 of age) were obtained from Johnson Laboratories (Chicago, IL) 1 day after birth. Forskolin was obtained from Calbiochem (San Diego, CA); testosterone was from Steraloids (Keene, NH); 17P-estradiol, GnRH, aprotinin, leucine-enkephalin, interleukin-lg was from Upstate Biotechnology Inc. (Lake Placid, NY). [3H]Chloramphenicol was obtained from Amer sham Corp., genistein, [3H]ATP, [3H]GTP, and [α-32P]dCTP were from ICN Biochemicals, Inc. (Costa Mesa, CA). The large EcoRI fragment of the mouse PGS-2 cDNA (15) was labeled and used to screen a chicken embryo fibroblast genomic DNA library (16). Out of approximately 300,000 plaques screened, eight positive clones >12 kb were identified and plaque-purified. One clone denoted 16-1 (described herein) was shown by restriction mapping to contain 7 kb of rat PGS-2 genomic sequences as well as 6 kb of 5'-flanking DNA (Fig. 1A). Two BamHI fragments of clone 16-1 were subcloned into pGEM3Zf(-), restriction mapped (Fig. 1B), and partially sequenced (Fig. 2).

Isolation of Rat PGS-2 Genomic DNA Sequences—A 5'-1.2-kb EcoRI fragment of the mouse PGS-2 cDNA (15) was labeled and used to screen a Chroma 35 phage library derived from partially digested rat liver DNA according to established procedures previously described (26). Out of approximately 300,000 plaques screened, eight positive clones >12 kb were identified and plaque-purified. One clone denoted 16-1 (described herein) was shown by restriction mapping to contain 7 kb of rat PGS-2 genomic sequences as well as 6 kb of 5'-flanking DNA (Fig. 1A). Two BamHI fragments of clone 16-1 were subcloned into pGEM3Zf(-), restriction mapped (Fig. 1B), and partially sequenced (Fig. 2).

Transformation and Extension Assays—To determine the transcription initiation site of the rat PGS-2 gene, primer extension analyses were performed (27). The RNA used in the reactions was either extracted from isolated intact preovulatory follicles incubated for 6 h with ovulatory levels of luteinizing hormone (500 ng/ml) and known to contain PGS-2 transcripts (20), or from corpora lutea obtained at day 18 of pregnancy, DNA preparation using the calcium phosphate precipitation technique (30, 31). Briefly, to prepare calcium phosphate precipitates, plasmid DNA (4.3 pmol) was combined with 17.5 μl of 2.5 M CaCl₂ and deionized H₂O to a final volume of 100 μl. The reaction was incubated at 37 °C for 20 min, ethanol precipitated, washed twice with 80% ethanol, dissolved in water, and used as a template for cDNA synthesis using the Superscript II reverse transcriptase. The reaction mixture contained 200 μM each of dNTPs, 2.5 μl of 10 X RT buffer (0.5 M Tris-Cl pH 8.2, 50 mM MgCl₂, 50 μM dithiothreitol, 0.5 M KCl, 0.5 mg/ml bovine serum albumin), 125 pM of RNasin, 18 μl of H₂O, and 40 units of avian myeloblastosis virus reverse transcriptase. The reaction was incubated at 37 °C for 20 min, with added chloroform and precipitated with alcohol, precipitated with 70% ethanol ethanol precipitated, washed twice with 80% ethanol, dissolved in water, and used as a template for primer extension. Each reaction contained 5 pmol of the primer and a template that contains rat genomic PGS-2 DNA spanning this region.

Primers were synthesized on a custom DNA synthesizer (Biosearch). Each reaction contained 5 pmol of each primer and a template that contains rat genomic PGS-2 DNA spanning this region.

Primer Extension Assays—To determine the transcription initiation site of the rat PGS-2 gene, primer extension analyses were performed (27). The RNA used in the reactions was either extracted from isolated intact preovulatory follicles incubated for 6 h with ovulatory levels of luteinizing hormone (500 ng/ml) and known to contain PGS-2 transcripts (20), or from corpora lutea obtained at day 18 of pregnancy, DNA preparation using the calcium phosphate precipitation technique (30, 31). Briefly, to prepare calcium phosphate precipitates, plasmid DNA (4.3 pmol) was combined with 17.5 μl of 2.5 M CaCl₂ and deionized H₂O to a final volume of 175 μl. This was added dropwise to an equal volume of 2 X HEPES-buffered saline (380 mM NaCl, 1.5 mM NaHPO₄, 50 mM HEPES, pH 7.2). Precipitates (350 μl) were added to granulosa cell cultures (3.5 ml of media) and incubated for 4 h at 37 °C. Cells were then washed twice with calcium/magnesium-free Hank's medium before being fed with fresh Dulbecco's modified Eagle's medium-Ham's F-12 supplemented with 5% fetal bovine serum, 10% horse serum, 10% dialyzed FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Under these conditions the granulosa cells differentiate to a preovulatory phenotype indistinguishable from that observed in vivo (20).

Transient Transfection Assay—Plasmid DNA was purified from bacteria by alkaline lysis and centrifugation on CaCl₂ gradients (26). Differentiated cultures of granulosa cells were transiently transfected with plasmids in a liposomal DNA precipitation technique (30, 31). Briefly, to prepare calcium phosphate-DNA precipitates, plasmid DNA (4.3 pmol) was combined with 17.5 μl of 2.5 M CaCl₂ and deionized H₂O to a final volume of 175 μl. This was added dropwise to an equal volume of 2 X HEPES-buffered saline (380 mM NaCl, 1.5 mM NaHPO₄, 50 mM HEPES, pH 7.2). Precipitates (350 μl) were added to granulosa cell cultures (3.5 ml of media) and incubated for 4 h at 37 °C. Cells were then washed twice with calcium/magnesium-free Hank's medium before being fed with fresh Dulbecco's modified Eagle's medium-Ham's F-12 supplemented with 5% fetal bovine serum, 10% horse serum, 10% dialyzed FBS, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

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A rat PGS-2 promoter CAT fusion construct (-2698/32PSG-CAT) was generated by isolating the genomic DNA fragment from -2698 to +32 (+1 = cap site), and fusing it upstream of the CAT gene in the vector pCATH-Basic (Promega) (Fig. 1D). To produce 5'-deletion mutants, the -2698/32PSG-CAT construct was digested with either SpI and SacI, SpI and SacI, and SacI and HindIII, or SpI and Bsr I. The DNA of interest was isolated after electrophoresis on 0.8% low-melt agarose, blunted ended when needed, ligated, and recombinants were isolated. These five different digests produced the fusion constructs -1968/32PSG-CAT, -628/32PSG-CAT, -604/32PSG-CAT, -192/32PSG-CAT, and -55/32PSG-CAT, respectively (Fig. 1D).

Nuclear Extracts—Extracts of nuclear proteins were prepared from

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**Rat PGS-2 Promoter**
granulosa cells of different developmental stages and from corpora lutea. Granulosa cells of immature follicles were collected from ovaries of untreated 26-day-old rats. To obtain granulosa cells of preovulatory stage follicles, hypophysectomized (H) rats (26 days of age) were primed with 17β-estradiol (E; 1.5 mg/day subcutaneously for 3 days) and follicle-stimulating hormone (FSH; F; 1.0 µg subcutaneously twice daily for 2 days), as previously described (20, 33, 34). The granulosa cells were isolated from the ovaries of these rats denoted HEF. Granulosa cells were also isolated 2 and 10 h after HEF rats had received an ovulatory dose of human chorionic gonadotropin (hCG, 10 IU intravenously); referred to as HEF+hCG 2 h and HEF+hCG 10 h. We have previously shown that rPGS-2 mRNA and protein are transiently induced in granulosa cells of HEF+hCG rats, with maximal levels reached at 4 and 5 h after hCG, respectively (20). Corpora lutea were obtained from adult rats on day 15 of pregnancy.

Nuclei were isolated and extracts were prepared as previously described (35, 36). Briefly, cells were homogenized (Teflon-glass homogenizer) in buffer (1.3 M sucrose, 10 mM Tris, 5 mM MgCl₂, 0.5% Triton X, 0.5 mM dithiothreitol) containing aprotinin (15 µg/ml), leupeptin (1 µg/ml), antipain (2 µg/ml), benzamidine (10 µg/ml), chymotrypsin (1 µg/ml), and pepstatin (1 µg/ml). The homogenate was filtered through siliconized glass wool, layered on 3 ml of buffer, and spun at 15,000 × g for 10 min at 4 °C. The pellet was resuspended in nuclear storage buffer (50 mM HEPES, pH 7.6, 3 mM MgCl₂, 0.1% EDTA, 25% glycerol, 10 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride) and stored at -80 °C. After thawing, nuclei were lysed by addition of 2 volumes of nucleic lysis buffer (2 mM MgCl₂, 100 mM KCl, 3 mM MgCl₂, 0.1% EDTA, and 12% glycerol), one tenth volume 4 M ammonium sulfate was added dropwise and then centrifuged at 100,000 × g for 1 h at 4 °C. The supernatant (nuclear extract proteins) was stored at -80°C until used in electrophoretic mobility shift assays. Protein concentrations were determined by the method of Bradford (Bio-Rad Protein Assay; 37).

Electrophoretic Mobility Shift Assay—To determine if proteins present in granulosa cell nuclear extracts were able to interact with a region (−192/−54) shown in transfection assays to confer forskolin-inducible expression of reporter gene activity, electrophoretic mobility shift assays were performed as described (38). Briefly, extracts of nuclear proteins (1.5-2 µg/reaction) were incubated with 5,000-10,000 counts/min of end-labeled DNA fragment (−192/−54) and 5 µg of poly(dI-dC) in a final volume of 20 µl in buffer containing 100 mM KCl, 15 mM Tris-HCl, pH 7.5, 5 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl₂, and 12% glycerol. Binding reactions were resolved by 5% polyacrylamide gels. To determine the specificity of protein-DNA interactions, binding reactions were also incubated in the presence of cold competitor DNA (used at 5-, 10-, and 25-fold molar excess). To further delineate the region within −192/−54 that was involved with protein-DNA binding, subfragments (−192/−109) and −109/−54 were generated by digesting the −192/−54 fragment with AluI, purifying the DNA subfragments, and using each as unlabeled DNA competitor.

RESULTS

Isolation of Rat PGS-2 Genomic DNA Sequences—A summary of restriction mapping and subcloning of clone 16-1 is depicted in Fig. 1. Clone 16-1 (as well as seven others; data not shown) were digested with BamHI and SacI, and restriction fragments were analyzed by Southern blots using the following 32P-labeled probes: the full-length 4.2-kb mPGS-2 cDNA, a 5' 1.2-kb, and a 3' 1.6-kb EcoRI fragment of the mPGS-2 cDNA, and two oligonucleotides corresponding either to the 5'-end (base pairs 176-205, 30-mer) or 3'-end (base pairs 3952-3982, 31-mer) of the mPGS-2 cDNA. As shown, clone 16-1 contains about 7 kb of rat PGS-2 genomic sequence, as well as about 6 kb of 5'-flanking DNA (Fig. 1A). Two BamHI fragments of clone 16-1 were digested with different enzymes for restriction mapping (Fig. 1B) and sequenced (Fig. 2). As shown in Fig. 1C, a PstI/BamHI subfragment (−6298/−376) was ligated upstream of a BamHI/SacI subfragment (−376/32), and subcloned into PGM3Zf(−) to generate a plasmid containing contiguous DNA sequences from −6298 to +32 of the rat PGS-2 gene. The DNA sequence is shown in Fig. 2. For transient transfection studies (see below), the fragment −6298/32 was subcloned upstream of the CAT reporter gene in the pCAT. Basic vector (Promega), and a series of 5'-deletion mutants were prepared using internal restriction sites as described under "Experimental Procedures."

Transcription Initiation Site—To map the transcription initiation site of the rat PGS-2 gene, primer extension analysis was performed using a 28-mer oligonucleotide complementary to base pairs 100-127 downstream of the transcription start site as identified in the mouse PGS-2 gene (Fig. 3A, 16). RNA extracted from preovulatory follicles incubated for 6 h with high levels of luteinizing hormone (500 ng/ml) is known to contain PGS-2 mRNA (20). When this RNA was used for primer extension, a 127-nucleotide extended product.
Rat PGS-2 Promoter

was obtained after comparison with an unrelated sequencing reaction run in adjacent lanes (Fig. 3, B and C). The size of the extended product was confirmed by comparison with a sequencing reaction containing the same oligonucleotide used for primer extension and a rPGS-2 genomic template spanning this region (data not shown). This result indicated the presence of a single transcription initiation site located 144 base pairs upstream of the predicted ATG translation initiation codon, which is highly similar to the cap site of the mouse PGS-2 gene (Fig. 3A). No extended product was detected when RNA from corpora lutea was used (Fig. 3C), as was expected because rPGS-2 mRNA is undetectable in this tissue.(39).

Functional Activity of Rat PGS-2 Promoter—To determine if the 5′-flanking region of the rat PGS-2 gene contains functional domains involved in its transcriptional regulation by hormones, a DNA fragment spanning from −2698 base pairs upstream to +32 downstream of the transcription start site was removed from its context and fused 5′ of the CAT reporter gene in the plasmid pCAT. Basic.(−2698/32PGS-CAT, Fig. 1D). Transient transfection of primary cultures of rat granulosa cells with the chimeric construct was used to test rPGS-2 promoter activity. We have previously shown that, in this primary cell culture system, rPGS-2 mRNA and protein are induced after 4–6 h of stimulation with ovulatory amounts of luteinizing hormone, FSH, or the agonist forskolin, a potent activator of adenylyl cyclase. Time course studies were first conducted to determine the optimal period of stimulation with forskolin (Fig. 4). Following a 4-h period of transfection, granulosa cells were cultured in the

![Fig. 2. Nucleotide sequence of the rat PGS-2 promoter from −2698 to +32. The arrowhead indicates the transcription initiation site (Fig. 3A).](image)

![Fig. 3. Identification of the transcription initiation site of the rat PGS-2 gene by primer extension analysis. A, nucleotide sequences of rat PGS-2 were aligned and compared to that of mouse PGS-2 from the transcription initiation site (open arrowhead) to the ATG translation initiation codon (closed arrowhead). For primer extension analysis, an anti-sense 28-mer oligonucleotide complementary to nucleotides 100–127 (boxed nucleotides) downstream of the mouse PGS-2 transcription initiation site was used. The key is: ---, for aligned identical bases; upper case letters, for aligned non-identical bases; lower case letters, for unaligned bases; . . . . , for nucleotide gaps. B, schematic representation of primer extension assay. The labeled anti-sense 28-mer oligonucleotide was hybridized RNA samples containing (preovulatory follicles incubated with luteinizing hormone) or not containing (corpora lutea) PGS-2 transcripts, and primer extension performed as described under "Experimental Procedures." A 127-nucleotide extended product was expected if rat and mouse PGS-2 were to have identical transcription initiation sites. C, products from primer extension assays were analyzed by electrophoresis on a 6% polyacrylamide, 7 M urea gel. The size of the extended product was determined by comparison with an unrelated sequencing reaction run in adjacent lanes and shown on the left. A single 127-nucleotide extended product (arrowhead) was obtained, and the oligo was hybridized to RNA from preovulatory follicles, whereas no extended product was detected RNA from corpora lutea was used.]
To test the ability of these peptides to activate PGS-2 gene activity, primary cultures of differentiated granulosa cells were transiently transfected with the chimeric construct −2698/32PGS-CAT (4.3 pmol), as described under "Experimental Procedures." Following CaPO4/DNA precipitation, cells were incubated for 3, 6, 9, 12, 24, 36, and 48 h in the presence or absence of forskolin (7.5 μM). Cells were then harvested, lysed, and the cytosolic fractions were assayed for CAT activity as previously described (32). Results are expressed as fold induction of CAT activity is forskolin-treated versus non-forskolin-treated cultures (mean ± standard deviations of duplicate cultures from two experiments).

absence or presence of forskolin for 3, 6, 9, 12, 24, 36, or 48 h. Results showed that maximal levels in CAT activity in the absence of forskolin (26% conversion) compared to the absence of forskolin (3.6% conversion) were reached after 6 h of stimulation. When expressed as fold induction of forskolin-treated versus non-treated cultures, a 7.2 ± 0.5-fold increase was observed at 6 h (Fig. 4). Thereafter, expression of CAT activity gradually declined, with little or no effect of forskolin detected at 36–48 h of culture (Fig. 4). Based on these results, we selected a 6-h stimulation period for all subsequent transient transfection experiments.

To delineate region(s) within the 2.7-kb DNA fragment involved in the forskolin-regulated activation of the rat PGS-2 promoter, a series of 5'-deletion mutants were designed using internal restriction sites (Fig. 1D). When these constructs were transiently transfected (4.3 pmol) in primary cultures of granulosa cells, results showed that deletions of up to 2.5 kb at the 5' end (i.e. from −2698 to −192) had no effect on the forskolin-stimulated reporter gene activity (4.2 ± 0.6 versus 3.5 ± 0.6-fold induction of CAT activity with −2698/32PGS-CAT and −192/32PGS-CAT, respectively, t test, p > 0.05; Fig. 5). However, significant increases in absolute levels of basal and forskolin-stimulated CAT activities were observed when the two constructs were compared (basal % conversion = 5.7 ± 1.6 versus 13.4 ± 1.5; forskolin-induced % conversion = 22.7 ± 5.1 versus 45.0 ± 7.6; t test p < 0.05, for −2698/32PGS6-CAT and −192/32PGS-CAT, respectively). Further deletion of the 5' end from −192/32PGS-CAT to −53/32PGS-CAT resulted in a marked loss in both basal (86% loss, t test p < 0.01) and forskolin-stimulated (90% loss, t test p < 0.01) reporter gene activities, clearly suggesting that the −192/-54 region contains key cis-element(s) involved in basal and forskolin-regulated expression of the rat PGS-2 gene.

Previous studies have shown that PGS-2 mRNA can be induced in differentiated granulosa cells in culture by luteinizing hormone, FSH, and GnRH, but not by interleukin-1β. To test the ability of these peptides to activate PGS-2 promoter activity, granulosa cell cultures were transiently transfected with the −2698/32PGS-CAT construct and challenged with the different agonists (Fig. 6). Results showed that FSH (500 ng/ml) and luteinizing hormone (500 ng/ml) were as efficient as forskolin for induction of reporter gene activity. In contrast, addition of GnRH or interleukin-1β had no significant effect on CAT activity when compared to control cultures (Fig. 6). Overall, similar results were observed when the same agonists were tested in cultures transfected with the shorter vector −192/32PGS-CAT (data not shown). These observations provided additional evidence that the first 206 base pairs upstream of the transcription start site comprised
a major regulatory region controlling transcriptional activation of the PGS-2 gene by luteinizing hormone, FSH, and forskolin.

**Rat PGS-2 Promoter Binding Activity**—The functional activity of the −192/−54 DNA fragment was further analyzed using electrophoretic mobility shift assays. For these assays, the end-labeled −192/−54 fragment was incubated with extracts of nuclear proteins prepared from ovarian cells isolated at different developmental stages. These included granulosa cells of immature follicles and of preovulatory follicles isolated before (t = 0 h) or after an ovulatory dose of hCG (t = 2 or 10 h), and corpora lutea collected from adult rats on day 15 of pregnancy. Multiple bands, indicative of protein-DNA interactions, were observed in each of the extracts. For reference purposes, the bands have arbitrarily been designated complexes I, II, III, and IV, as shown in Fig. 7. Some protein-DNA complexes (I and II) were present in all extracts, whereas others appeared developmentally regulated (complexes III and IV). Specifically, complexes I and II were observed in extracts of cells/tissue including those in which PGS-2 mRNA is not expressed (i.e. immature and HEF granulosa cells as well as corpora lutea). In contrast, complexes III and IV were only observed in nuclear extracts of granulosa cells prepared from preovulatory follicles collected 2 and 10 h after hCG (i.e. in cells in which PGS-2 mRNA and protein have been induced, respectively, 20).

To test the specificity of the protein-DNA complexes, competition assays were performed using molar excess (2-, 10-, and 25-fold) of unlabeled DNA fragments, the −192/−54 fragment as well two subfragments, −192/−110 and −110/−54. The nuclear extract chosen for these assays was that bearing temporal and thus presumably physiological relevance with regard to transcriptional activation of rPGS-2, i.e. HEF+hCG (2 h). Results showed all complexes (I-IV) exhibited binding that was specific and efficiently competed by the unlabeled −192/−54 fragment (Fig. 8). The ability of the −192/−110 subfragment, but not the −110/−54 subfragment, to inhibit binding suggests that the protein-DNA binding formed with the undigested −192/−54 fragment was located within the region −192/−110.

**DISCUSSION**

Collectively, the results presented in this study provide the first structural and functional evidence that transcriptional regulation of the rat PGS-2 gene by gonadotropins in granulosa cells involves 5'-flanking DNA sequences. Using a fragment of the mouse PGS-2 cDNA as a probe, we isolated and characterized a rat genomic clone containing up to 6 kilobases of upstream sequences of the rat PGS-2 gene. Comparisons between rat and mouse PGS-2 promoters revealed a high degree of homology, whereas the promoter of the chicken PGS-2 gene appears dissimilar (22). The transcription initiation site of mouse and rat PGS-2 was identical and nucleotide

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**FIG. 7.** Identification and developmental regulation of DNA binding activities in nuclear extracts of rat ovarian cells. Extracts of nuclear proteins were prepared from granulosa cells of different developmental stages and from corpora lutea. Immature granulosa cells were collected from 26-day-old rats, while granulosa cells of preovulatory stage follicles were isolated from hypophysectomized (H) rats primed with 17β-estradiol (E) and FSH (F; nuclear extract denoted HEF). Extracts were also prepared 2 and 10 h after HEF rats had received an ovulatory dose of hCG (HEF + hCG 2 h, HEF + hCG 10 h extracts), and from corpora lutea obtained from adult rats on day 15 of pregnancy. Extracts were incubated with the 32P-labeled DNA fragment −192/−54, and protein-DNA interactions were tested in electrophoretic mobility shift assays as described under “Experimental Procedures.” Binding reactions were resolved by 5% acrylamide, 0.5 × TBE gel electrophoresis. For reference purposes, the multiple protein-DNA interactions were designated complexes I-IV.

**FIG. 8.** Protein-DNA binding specificity with the granulosa cells nuclear extract “HEF + hCG 2 h” and the 5'-DNA fragment −192/−54. To test for protein-DNA binding specificity, competition assays were performed by incubating the HEF+hCG (2h) extracts and the 32P-labeled fragment −192/−54 with molar excess (2-, 10-, and 25-fold) of unlabeled DNA fragments. A, the unlabeled DNA fragments included cold −192/−54, as well as two subfragments (−192/−110 and −110/−54) generated by digesting −192/−54 with AluI. B, binding reactions were resolved by 5% acrylamide, 0.5 × TBE gel electrophoresis.
sequences located within 1 kb upstream of the cap site exhibited an overall identity of 83% (17). The rat promoter contained no consensus TATA box motif, although the hexanucleotide TTAAAA, described as a presumptive TATA element in the mouse promoter (17), was conserved 30 base pairs upstream of the transcription start site in the rat. Primer extension analyses suggested that the transcription initiation site is stringently controlled, with only a single extended product detected. This contrasts with promoters of other genes lacking the consensus TATA-box motif which exhibit multiple transcriptional initiation sites. Included in this category of genes are the promoters for RII (the regulatory subunit of type II CAMP-dependent protein kinase, 31), the luteinizing hormone receptor (40, 41), and progesterone receptor (42), all of which are expressed in a hormonally dependent manner in rat ovarian granulosa cells (for a recent review, see Ref. 43).

The recent development and characterization of a primary culture system of differentiated granulosa cells (20, 29) allowed us to test under optimal conditions the promoter activity of rPGS-2 5'-flanking DNA. In this system, immature granulosa cells are differentiated in vitro to a developmental stage equivalent to that of granulosa cells of preovulatory follicles (20). Most importantly, we have previously shown that induction of rPGS-2 mRNA and protein in preovulatory follicles in vivo by an ovulatory dose of gonadotropins could be reproduced in vitro in the primary cultures with high levels (500 ng/ml) of luteinizing hormone or FSH (20). In this respect, it is important to note that, following transient transfection of primary granulosa cell cultures with the −2698/32PGS-CAT fusion construct, the time course induction of reporter gene activity by gonadotropins and forskolin closely mimicked that expected for induction of endogenous PGS-2 mRNA and protein (20). The transient transfections indicate further that the presence of the first 192 base pairs upstream of the transcription initiation site are sufficient to confer gonadotropin- and forskolin-activated transcription of the reporter gene. The increase in basal activity obtained with the construct −192/32PGS-CAT, as compared to that obtained with the longer −2698/32PGS-CAT, may result from the removal of negative 5'-regulatory elements, as also suggested by Fletcher et al. (17) following transfection studies of NIH 3T3 fibroblasts with mouse PGS-2 promoter/luciferase gene chimeric construct. However, the present study clearly documents that removal of an additional 139 base pairs (i.e. use of −53/32PGS-CAT) causes a dramatic loss in basal and forskolin-regulated promoter activities, thereby establishing the region between −192 and −54 as an essential component for the transcriptional regulation of the rPGS-2 gene by hormones. The inability of GnRH to induce reporter gene activity was unexpected because the decapetide has been shown to be as effective as luteinizing hormone and FSH at inducing rPGS-2 mRNA and protein in vitro (20, 44). Although it is unclear why GnRH had no stimulatory effect on the reporter construct, it remains possible that cis-acting DNA elements necessary for transducing GnRH action reside outside the 2.7 kilobases of 5'-flanking DNA present in the fusion construct or that factors of the GnRH signaling pathway were impaired by the transfection procedure.

The cis-acting DNA elements and trans-acting factors which are known to mediate cAMP activation in other genes include the CAMP-responsive element (CRE; 5'-TGACGTCA-3', 45) and its binding proteins of the CREB family, as well as activator protein 2 element (AP-2; 5'-CCCCAGGC-3', 46). Within 2.7 kb of 5'-flanking sequence of the PGS-2 gene, including the cAMP-regulated promoter region between −192/−54, there are no consensus CRE or AP-2 sites. The absence of these elements in cAMP/gonadotropin-regulated genes is not without precedence because the promoters of several other genes (cytochromes P450, and P450, luteinizing hormone receptor, and RII) which are known to be transcriptionally regulated by gonadotropin in granulosa cells lack these functional sequences (31, 40, 47, 48). Therefore, transcriptional factors, in addition to members of the CREB/ATF family and AP-2, appear to be involved in cAMP-regulated expression of genes in these endocrine cells. The presence of an AP-1-like element (5'-TGAGTCA-3', 45) at position −165/−159 of the PGS-2 promoter places this within the regulated promoter sequence and may account for inducibility by agonists which activate protein kinase C and transcription factors such as jun/fos. Although Sp-1 sites (5'-GGGCCGG-3', 49) have been identified in the mouse PGS-2 promoter (17) and suggested as a possible regulatory element, the Sp-1 site in the rat PGS-2 promoter lies outside of the regulatory region (−192/−54) at position −238/−233. Thus, it is unlikely that Sp-1 plays a major role in mediating cAMP-inducible activity.

The binding of trans-activating nuclear proteins to specific cis-acting DNA elements represents a central step in the regulation of gene transcription (50, 51). Attempts to demonstrate specific binding between proteins present in granulosa cell nuclear extracts and the −192/−54 DNA fragment provide the first evidence that the ovarian extracts contain proteins capable of such interactions. The complex banding pattern observed in electrophoretic mobility shift assays combined with competition analyses is suggestive of multiple protein-DNA interactions, with binding occurring most likely to sequences within −192 and −110 of the transcription start site. Although the functional relevance of these interactions remains to be clearly established, one could hypothesize that the complexes (I and II) detected using nuclear extracts of all developmental stages include factors that, though binding to DNA, cannot by themselves initiate transcription. In contrast, the additional protein-DNA complexes (III and IV) observed with granulosa cell nuclear extracts prepared after administration of an ovulatory dose of gonadotropins could reflect de novo synthesis and the binding of factors directly involved in transcriptional activation of the PGS-2 gene. Alternatively, the administration of gonadotropins may cause post-translational modifications (i.e. phosphorylation or others) of existing nuclear factors, thereby altering their binding affinity to DNA or to other nuclear proteins (52). Future studies should delineate the precise sites of these interactions within the −192/−110 fragment, and combined with site-directed mutagenesis and transient transfection assays, should determine if the observed physical interactions of proteins and DNA relate to functional transcriptional activation and involve the AP-1 site or additional cis-acting elements. In summary, the ability of primary cultures of differentiated granulosa cells to closely reproduce in vitro the induction by gonadotropins of rPGS-2 protein provides a unique model to study the transcriptional regulation of the PGS-2 gene in the physiological context of the ovulation process.

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