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GATA-4 and GATA-6 Transcription Factors: Expression, Immunohistochemical Localization, and Possible Function in the Porcine Ovary[1]

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ABSTRACT

The expression and localization of GATA-4 and GATA-6 mRNAs and proteins were assessed in porcine ovaries at different stages of the estrous cycle. Reverse transcription polymerase chain reaction and Western blot analyses revealed that GATA-4 and GATA-6 transcripts and proteins were strongly expressed in granulosa cells isolated from antral follicles, intact antral follicles, corpora hemorrhagica (CH), and midluteal phase corpora lutea (CL). Immunoblot analyses showed two predominant proteins with molecular masses of approximately 53 and 55 kDa for GATA-4 and one 55-kDa protein for GATA-6. Immunohistochemical studies revealed GATA-4 and GATA-6 nuclear staining in granulosa cells of healthy primordial and primary antral follicles and antral follicle of various sizes. The percentage of immunopositive thecal cell nuclei increased with follicular development. In CH and CL, luteal cells displayed nuclear immunoreactivity for both transcription factors. Regressing CL showed a decrease in GATA-immunopositive cells. Immunoreactivity for GATA-4 and GATA-6 was present in most blood vessels. In electrophoretic mobility shift assays, nuclear protein extracts isolated from granulosa cells and CL exhibited both GATA-4 and GATA-6 binding to a GATA consensus oligonucleotide, with GATA-4 the predominant binding protein. GATA-4 and GATA-6 DNA binding was elevated in granulosa cell nuclear extracts from preovulatory (8–10 mm) follicles. Cotransfection of primary cultures of luteinizing granulosa cells with GATA-4 or GATA-6 expression vectors increased the activity of the porcine steroidogenic acute regulatory protein gene promoter significantly but did not significantly activate the inhibin α gene promoter. The detection of GATA-4 and GATA-6 mRNAs and proteins in porcine ovaries and the identification of at least one possible target gene may help to establish roles for these GATA factors in follicular development and luteal function.

corpus luteum, follicle, gene regulation, granulosa cells, ovary

INTRODUCTION

The GATA family proteins are a group of zinc finger transcription factors that play an important role during mammalian organ morphogenesis, cell proliferation, and sex differentiation [1]. These factors bind to a consensus DNA motif (A/T)GATA(A/G) in the promoters of target genes in a variety of tissues [1]. Although they appear to exhibit similar DNA-binding properties, GATA family proteins have diverse expression patterns. Their specificity appears to be modulated by protein interactions with other transcription factors. Six GATA family members have been identified in vertebrates and can be divided into two groups based on their tissue distribution and homology: GATA-1, -2, and -3 and GATA-4, -5, and -6 [2]. GATA-1, -2, and -3 genes are predominantly expressed in hematopoietic cells where they are involved in proliferation and differentiation of several cell lineages [3–7]. GATA-4, -5, and -6 are expressed in heart, liver, lungs, and the gastrointestinal tract where they mediate tissue-specific gene expression [8–10]. In the endocrine system, GATA-4 and GATA-6 are expressed in the hypothalamus [11], pituitary [12], male and female gonads [13–16], and adrenal glands [17].

During murine gonadal development, GATA-4 and GATA-6 appear to play an important role in the regulation of testis-specific gene expression. GATA-4 expression is maintained in Sertoli cells throughout embryonic development, whereas it is downregulated after histological differentiation of the ovary on embryonic Day 13.5, indicating a role in early development and sexual dimorphism [15]. A recent study has shown that GATA-4 and GATA-6 transcripts are present in late fetal, neonatal, juvenile, and adult Sertoli cells of mouse testis, and GATA-4 protein was present in the nuclei of Sertoli and Leydig cells in postnatal animals [13].

GATA-4 and GATA-6 have been identified in the adult ovaries of humans, mice, and chickens [9, 14, 16]. In mouse ovary, GATA-4 mRNA has been localized to granulosa cells of primary and early antral follicles, with smaller amounts in thecal and interstitial cells. Little or no GATA-4 mRNA was found in mouse CL, whereas GATA-6 mRNA was highly expressed [14]. In adult mice, strong expression of GATA-6 mRNA was found in granulosa cells, but it was not evident in theca, interstitial cells, or oocytes [14]. In humans, GATA-4 and GATA-6 transcripts and GATA-4 protein have been localized in granulosa and thecal cells of both preantral and antral follicles of normal ovaries and tumor cell lines [16].

Of the few studies concerning the gonads, most have focused on the localization of GATA-4 and GATA-6. Little is known about the possible role of these factors in reproductive function; however, recent functional studies have implicated GATA-4 in gonadal gene regulation [18–20]. Cotransfection of a mouse GATA-4 expression vector with promoters for the murine Müllerian inhibiting substance, aromatase, steroidogenic acute regulatory protein (StAR), or inhibin-α genes resulted in transactivation in clonal cells [13, 18]. Virtually nothing is known about GATA-6 target genes in gonads or whether GATA-4 or GATA-6 can activate putative target genes in primary ovarian cells.

In one study, the temporal and spatial localization of

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MYQSLAMAANHGPPPGPYEAGGPGPFMHSAGAASSPVYVPTPRVPSSVLG    50

LSYLQGGGGGAAAGAASGGSSSAAPSGAGPGTQQGSPGWSQAGAEGAAYT   100

PPPVSPRFSFPGTTGSLAAAAAAAAAREAAAYSSGGGAAGAGLAGREQYG   150

RAGFAGSYSSPYPAYMADVGASWAAAAAASAGPFDSPVLHSLPGRANPAA   200

RHPNLDMFDDFSEGRECVNCGAMSTPLWRRDGTGHYLCNACGLYHKMNGI   250

SRPLIKPQRRLSASRRVGLSCANCQTTTTTLWRRNAEGEPVCNACGLYMK   300

LHGVPRPLAMRKEGIQTRKRKPKNLNKSKTSAGPSGSESLPPTTSASSNS   350

SSVATSSSEEMRPIKTEPGLSAHYGHGSSLSQTFSVSAMSGHGPSIHPVL   400

SALKLSPQGYASSVSQSPQASSKQDPWNSLALADSHGDIITA*          442
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FIG. 1. Predicted translation of the full-length porcine GATA-4 cDNA (GenBank accession no. AY115491). The predicted GATA-type zinc finger domains, amino acids 211–265 and 265–318, are underlined, and critical cysteine residues are shown in bold.

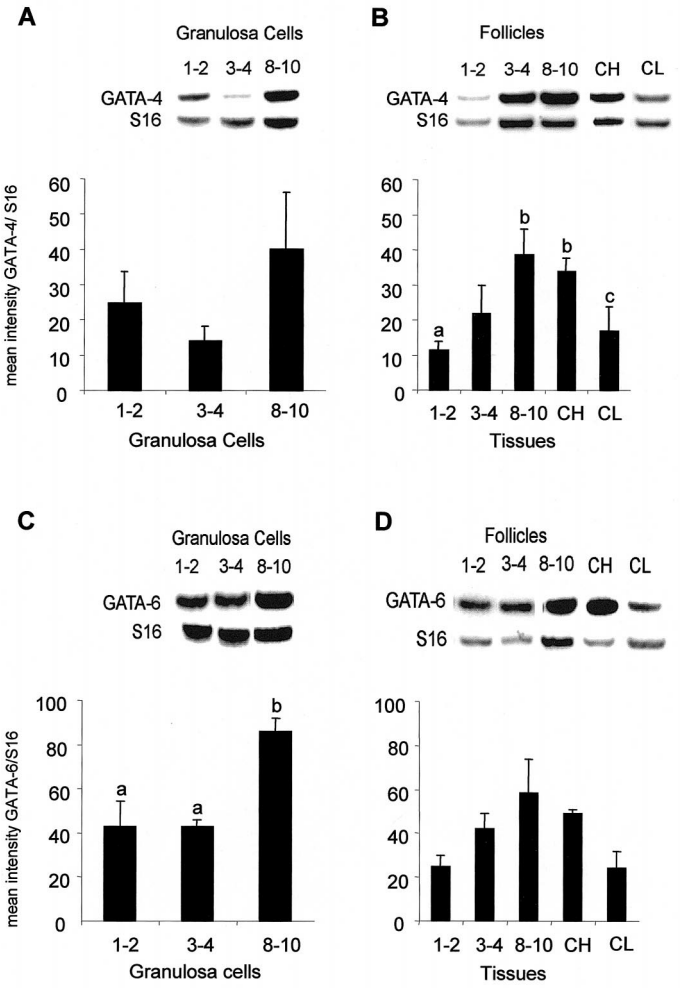


FIG. 2. Detection of GATA-4 and GATA-6 transcripts in porcine ovaries by RT-PCR. Representative autoradiograms for a single group of cells/structures is shown for coamplified GATA-4 and S16 cDNAs (**A** and **B**) or GATA-6 and S16 cDNAs (**C** and **D**). Graphs represent the mean ratios of GATA-4:S16 (**A** and **B**) or GATA-6:S16 (**C** and **D**) optical densities ± SEM for three independent isolations of RNA. In **A** and **C**, numbers represent granulosa cells isolated from antral follicles of 1–2, 3–4, and 8–10 mm. In **B** and **D**, numbers represent the diameter of the antral follicles (mm) isolated. Also shown are CH and CL. Within each graph, different letters represent significant differences between groups ($P \leq 0.05$, Fisher PLSD test).

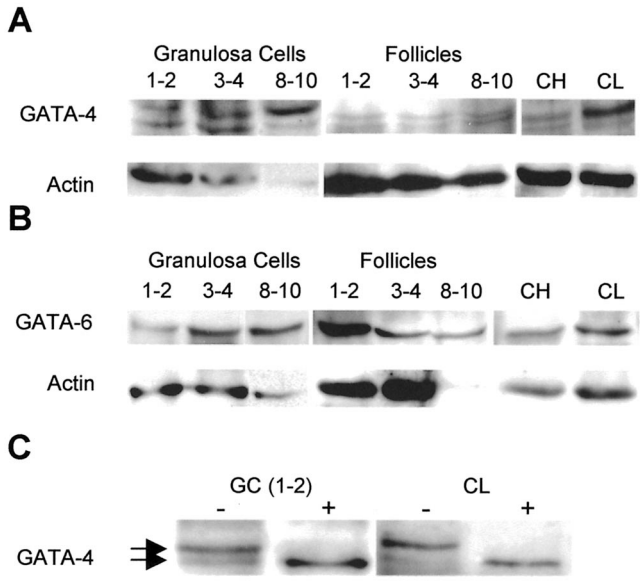


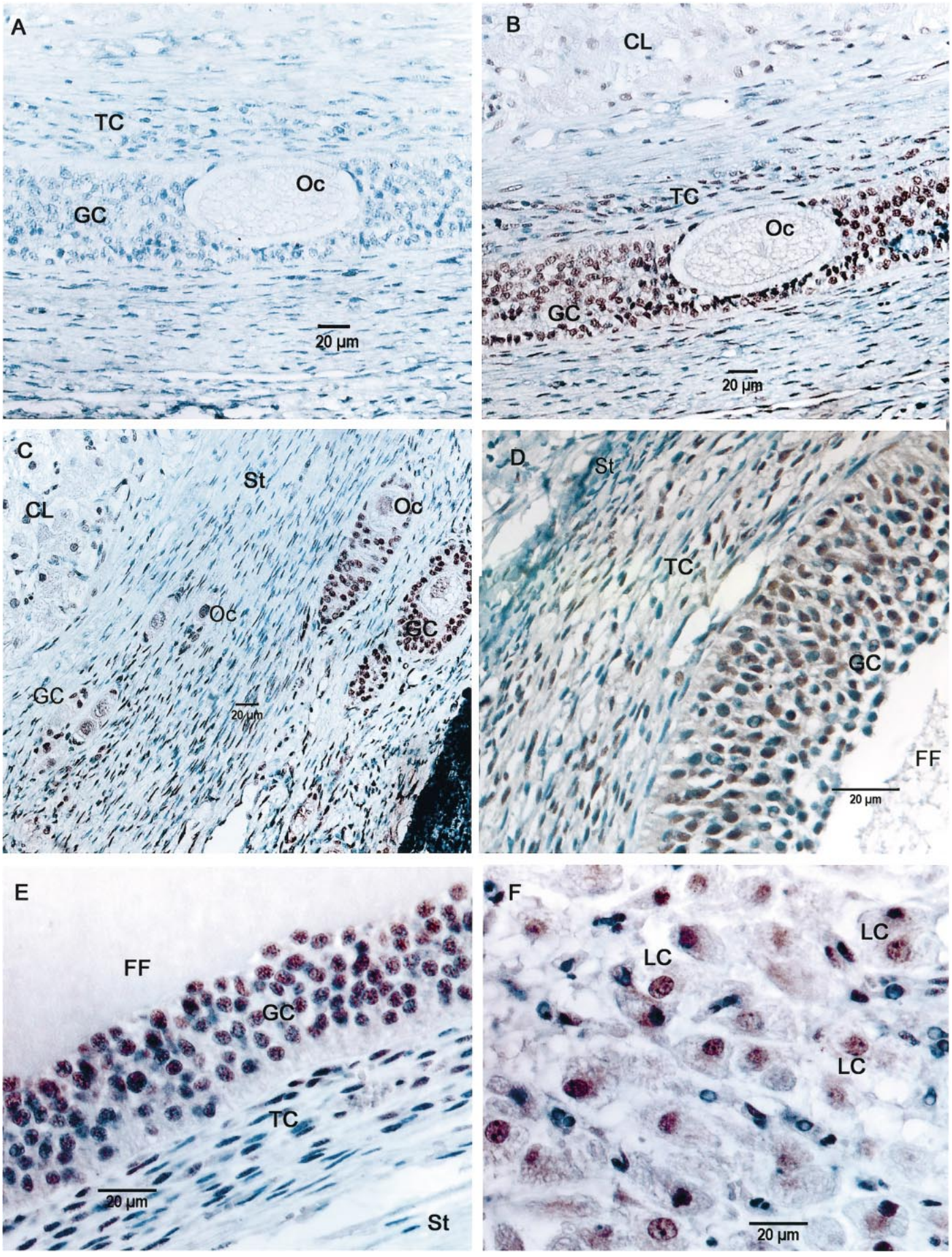
FIG. 3. GATA-4 and GATA-6 protein expression in porcine ovarian structures. Whole cell extracts (50 μg) from granulosa cells isolated from antral follicles of 1–2, 3–4, and 8–10 mm or follicles of the indicated diameter (mm), CH, and midluteal phase CL were separated by SDS-PAGE followed by immunoblotting for GATA-4 (**A**) or GATA-6 (**B**). The relative molecular masses of the proteins shown in this figure were 53 and 55 kDa for GATA-4, 55 kDa for GATA-6, and 43 kDa for actin. **C**) Detection of phosphorylated GATA-4 protein by immunoblot analysis in granulosa cells and CL. The λ-phosphatase treatment (+) of nuclear extracts resulted in a shift in molecular mass of GATA-4 compared to untreated (−) samples. GC (1–2) indicates granulosa cells isolated from 1- to 2-mm antral follicles.

GATA-4 protein was evaluated during gonadogenesis in pigs [21]. In that study, GATA-4 protein was present in the bipotential gonad in both female and male embryos. During fetal and postnatal development, GATA-4 was present in Sertoli and Leydig cells of the testis and in somatic cells of the differentiating ovary. The authors suggested that GATA-4 could have an important role in development of both XX and XY porcine gonads [21]. There are no published reports of the localization or possible functions of GATA-4 and GATA-6 in the ovaries of cycling pigs. Here, we report the cloning of the porcine GATA-4 coding sequence and the expression and localization of GATA-4 and GATA-6 messages and proteins in porcine ovaries at different stages of the estrous cycle. We also analyzed cyclic changes in GATA-4 and GATA-6 DNA-binding activities to a GATA consensus oligonucleotide and tested the abilities of GATA-4 and GATA-6 to activate two ovarian gene promoters with known GATA sequences, StAR and inhibin-α, in transfected primary ovarian cell cultures.

MATERIALS AND METHODS

Materials

General chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. Cell culture reagents, Trizol, LipofectAMINE, oligonucleotides, and the pCRII and pcDNA3.1 plasmids were purchased from Invitrogen (Carlsbad, CA). The pALuc plasmid was a gift from Dr. Richard Day (University of Virginia, Charlottesville, VA). Moloney murine leukemia virus H+ and H− reverse transcriptases, random hexamers, restriction enzymes, Rnasin, and T4 polynucleotide kinase were obtained from Promega Corp. (Madison, WI). *Taq* polymerase (for semiquantitative reverse transcription polymerase chain reaction) was purchased from Fisher Scientific (Fair Lawn, NJ). Radioisotopes were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). All primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Plasmid pu-



rification reagents were obtained from Qiagen (Valencia, CA). Porcine ovaries were obtained from a local abattoir.

Cloning of GATA-4 cDNA and Plasmid Constructs

The entire coding sequence for porcine GATA-4 had not been previously cloned and was isolated by reverse transcription and polymerase chain reaction (RT-PCR). The downstream GATA-4 primer (5′-CAGGAATCTGAGGAGGGAAGGAGGTGAC-3′) was derived from the 3′untranslated region of a partial porcine cDNA, GenBank accession no. AF190704. The upstream primer was derived from human GATA-4 accession no. L34357 (5′-ATGTATCAGAGCTTGGCCATGGCCGCCAACC-3′). The amino acid sequence in this region is homologous among rats, mice, and humans. The GATA-4 coding sequence was amplified from cDNA generated from two independent pools of porcine ovarian poly(A) RNA using either the Marathon cDNA kit or the SMARTRACE kit (Clontech, Palo Alto, CA). This porcine GATA-4 sequence has been submitted to GenBank (accession no. AY115491). The GATA-6 coding sequence was also isolated by RT-PCR using primers derived from GenBank accession no. AF295687. The oligonucleotide primers were 5′-GAGCAGCCGGAGGAGATGTACCAGAC-3′ and 5′-GGCTCAGGCCAGGGCCAGGGCGCACC-3′. GATA-6 was amplified using cDNA synthesized from ovarian poly(A) RNA using the Marathon cDNA kit (Clontech). Both of the sequences have high GC content, and PCRs were performed using the Advantage GC cDNA PCR kit (Clontech). The cDNAs were ligated into the pCRII vector for sequence verification and then subcloned into the pcDNA3.1 expression vector (Invitrogen). DNA sequencing was also performed to confirm insert orientation (DNA sequencing facility, University of Maine, Orono, ME).

The pStARLuc plasmid, containing 1544 base pairs (bp) of the 5′ flanking region of the porcine StAR gene, and the promoterless luciferase vector pALuc have been previously described [22, 23]. The porcine inhibin-α subunit promoter has not been previously isolated. A 363-bp fragment of the porcine inhibin-α gene 5′ flanking region containing two GATA sites was amplified from two independent pools of porcine genomic DNA using the Advantage II polymerase and PCR kit (Clontech). The upstream oligonucleotide primer was derived from the bovine promoter, accession no. S72864, and includes a *Kpn*I site at the 5′end (5′-GGGGTACCGTCCCATCTGGTGTCACCTCTG-3′). This primer region is highly homologous between human and bovine sequences. The downstream oligonucleotide was derived from porcine inhibin-α cDNA, accession no. X03265, and includes an *Sal*I site added at the 5′ end (5′-GGGTCGACGCCAGGTGAGCTATGTGGCCT-3′). The amplified product was subcloned into the *Kpn*I-*Sal*I sites of the pALuc vector and sequenced as above. This porcine inhibin-α promoter sequence has been submitted to GenBank, accession no. AF510728. Sequence comparisons were performed using BLAST alignment [24]. Structural analysis of the GATA-4 predicted translation product utilized the PROSITE database [25].

Semiquantitative RT-PCR

Semiquantitative RT-PCR was utilized to detect GATA-4 and GATA-6 transcripts. RNA was isolated using Trizol reagent from granulosa cells (from antral follicles 1–2, 3–4, and 8–10 mm), small follicles (1–2 mm), medium follicles (3–4 mm), healthy preovulatory large follicles (8–10 mm), corpora hemorrhagica (CH), midluteal phase corpora lutea (CL), and regressive CL dissected from ovaries of cycling pigs. CH, midluteal CL, and regressive CL were classified based on gross morphological appearance, including size, extent of vascularity, presence or absence of central blood clot, color, and overall appearance of ovary as described by Akins and Morrissette [26]. CH used in this study were estimated to be Day 4, midluteal phase CL were from Days 9–12, and regressive CL were from Days 19–21 of the estrous cycle. Ten micrograms of RNA for each sample was treated with RNase-free DNase I (DNA-*free* kit; Ambion, Austin, TX) for 30 min at 37°C followed by re-extraction with Trizol reagent or DNase Inactivation reagent (DNA-*free* kit). Three micrograms of DNase-treated RNA was reverse transcribed. PCR was carried out using 5 μl of each cDNA reaction as previously described [27] with the modification of 120 μM dNTPs (Clontech) added during the PCR. RNA concentrations were measured spectrophotometrically prior to and after DNase treatment. For PCR, the oligonucleotide primers 5′-ATGAAGCTCCATGGTGTCCC-3′ and 5′-ACTGCTGGAGTTGCTGGAAG-3′ corresponding to porcine GATA-4 nucleotides 895–914 and 1056–1037, respectively, were used. For GATA-6, 5′-AGAAACGCCGAGGGTGAAC-3′ and 5′-CGTTTCCTGGTCTGAATTCCC-3′ corresponding to porcine nucleotides 922–940 and 1031–1011, respectively, were used for PCR. Porcine ribosomal protein S16 was used as an internal control as previously described [27]. Amplification conditions consisted of denaturation at 94°C for 2 min followed by 25 cycles of 94°C for 30 sec, 48°C for 45 sec, and 72°C for 1 min, with a final extension of 72°C for 7 min. This cycle number was determined to be in the linear range for each primer set in preliminary experiments. A water control was carried through all reactions. Five microliters of each PCR sample was electrophoretically separated on 6% acrylamide gels. Dried gels were exposed to X-OMAT film with intensifying screens for 3–5 h at −70°C. Autoradiographic bands were quantified by Scion Image software (Scion Corp., Frederick, MD). Optical density units of GATA-4 and GATA-6 cDNA autoradiographic bands were normalized with the corresponding S16 cDNA. Expected products were 159 bp for GATA-4, 110 bp for GATA-6, and 196 bp for porcine S16 cDNA.

To verify the identity of GATA PCR products, nonradioactive reactions were amplified in parallel experiments, and PCR products were separated on 1% agarose gels (data not shown). PCR bands were gel purified, subcloned into the pCRII vector (Invitrogen), and sequenced.

Protein Extraction and Immunoblot Assays

Whole cell lysates were prepared, and immunoblotting was performed as previously described [27]. After isolation, protein concentrations were determined using Bio-Rad dye reagent (BioRad Laboratories, Hercules, CA). Equivalent amounts of protein (50 μg) were separated by 10% SDS-PAGE minigels for approximately 100 min at 200 V to elongate the region between 50 and 60 kDa and were then electrotransferred to membranes. Blocked membranes were incubated with 4 μg/ml anti-GATA-4 (H-112X), 2 μg/ml anti-GATA-6 (H-92X), 1 μg/ml anti-actin (H-300), or normal rabbit IgG (2–4 μg/ml) in Tris-buffered saline supplemented with 0.05% of Tween-20 (TTBS) plus 1% nonfat milk overnight at 4°C. The GATA-4 (H-112) antibody recognizes the first 112 amino acids of recombinant human GATA-4, whereas the GATA-6 antibody (H-92) recognizes the first 92 amino acids of recombinant human GATA-6. No blocking peptides are available for these antibodies. Following extensive washing with TTBS, membranes were incubated with a 1:1500 dilution of horseradish peroxidase goat anti-rabbit IgG secondary antibody (65-6120; Zymed, South San Francisco, CA) in TTBS plus 5% milk for 1 h at room temperature. After extensive washing with TTBS, immunoreactive bands were detected by enhanced chemiluminescence (Amersham). Membranes were reprobed for actin.

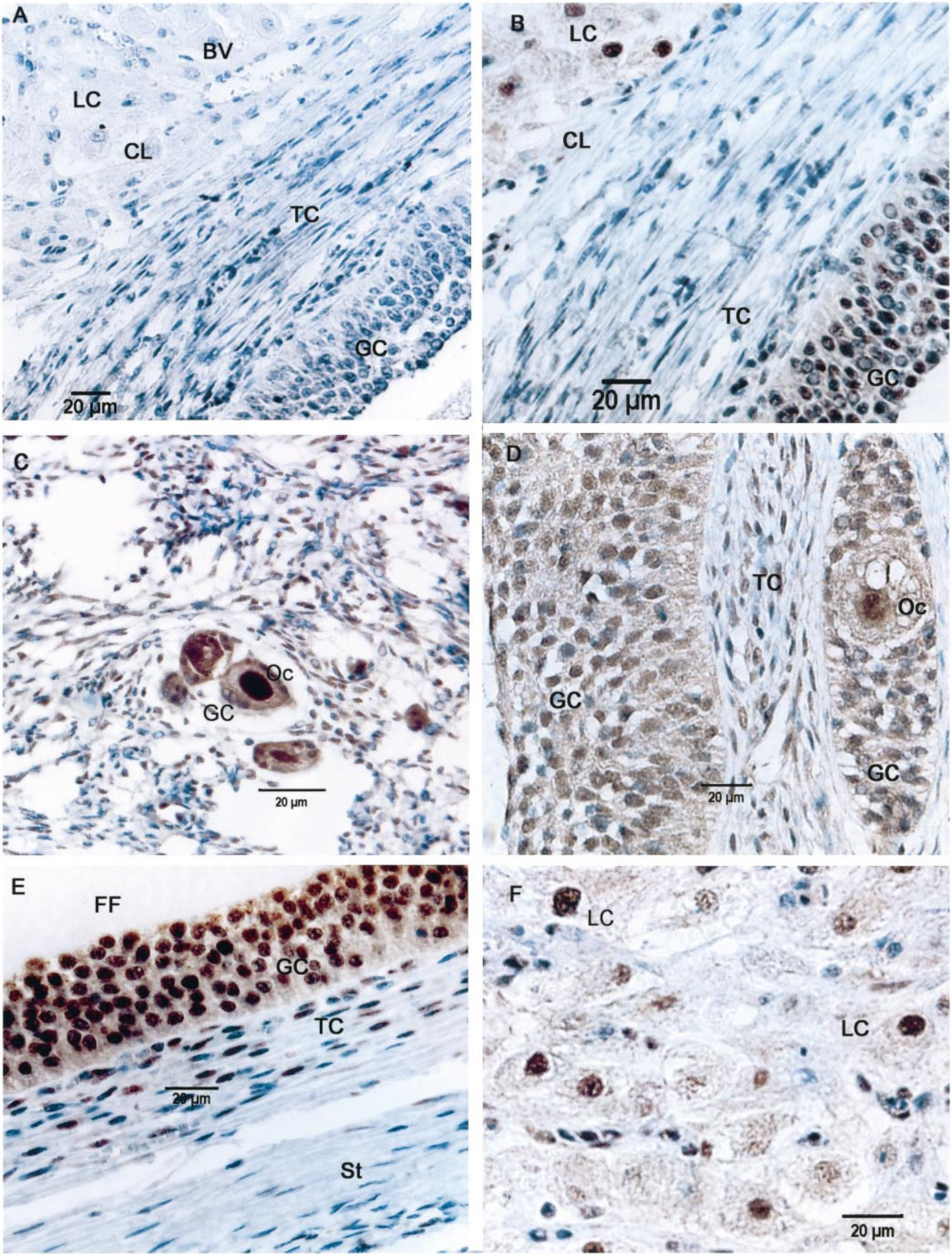
To determine the presence of phosphorylated and nonphosphorylated forms of GATA-4 or GATA-6, nuclear extracts from granulosa cells of 1- to 2-mm antral follicles and CL were isolated as previously described [27], with the exception that 1 mM tetrasodium pyrophosphate, 0.1 mM $NaVO_3$, and 1 mM NaF were added to buffers A and C. Nuclear extracts (40 or 50 μg) were incubated with 2500 units/ml λ-phosphatase (λ-PPase; New England BioLabs, Beverly, MA) at 30°C for 30 min followed by electrophoresis as described above.

Immunohistochemistry

Immediately following evisceration, porcine ovaries were placed in 4% paraformaldehyde. After transport to the laboratory, ovaries were cut into 3- to 5-mm-thick sections for continued fixation in 4% paraformaldehyde. Samples were paraffin embedded using standard methodology. Approximately 8-μm serial sections were prepared and placed on poly-L-lysine-coated microscope slides. Sections were deparaffinized in xylene, rehydrated through a series of ethanol washes, and rinsed in water.

Antigen retrieval was performed according to a heat-induced epitope retrieval procedure (Zymed) [28–30]. Sections were immersed in 0.01 M citrate buffer solution, pH 6.0, and subjected to microwave heating for 20 min at 800 W followed by 15 min at 300 W in a microwave pressure cooker (Nordic Ware, Minneapolis, MN). The sections were allowed to

FIG. 4. Immunohistochemical localization of GATA-4 protein in porcine ovarian structures. Positive staining is shown in brown. Ovaries were stained with GATA-4 antibody and secondary antibody. In each slide, granulosa (GC), theca (TC), stromal (St), and luteal (LC) cells and oocytes (Oc) are indicated. Bars = 20 μm. **A**) Section of ovary including a primary follicle. IgG was used in place of the primary antibody (control slide). **B**) Same as section **A** but in the presence of the GATA-4 primary antibody. **C**) Section of ovary including CL and primordial (left) and primary (right) follicles. **A**–**C**) Magnification ×20. **D**) Section of ovary including a 3- to 4-mm antral follicle. **E**) Section of ovary including a 7-mm antral follicle. **F**) Section of ovary including a midluteal phase CL. **D**–**F**) Magnification ×40.



cool for 10–15 min, and sections were then washed in water (5 min) followed by a wash for 10 min with Dulbecco PBS (D-PBS) at room temperature. Endogenous peroxidase activity was blocked by incubating sections in 0.3% H_2O_2 in methanol for 40 min at room temperature. Slides were washed in D-PBS (5 min), then the sections were blocked for 1 h in D-PBS supplemented with 10% normal goat serum. Localization of GATA family proteins was performed by incubation with either 5 μg/ml rabbit polyclonal GATA-4 (H-112X) or 1 μg/ml rabbit polyclonal GATA-6 (H-92X) at 4°C overnight. Negative controls for each procedure included replacement of GATA primary antibody with the same concentration of normal rabbit IgG. After washing in D-PBS, sections were incubated with 1:500 horseradish peroxidase-conjugated goat anti-rabbit IgG (65-6120; Zymed) in 10% goat serum for 1 h at room temperature. To visualize bound antibody, the 3,3′-diaminobenzidine tetrahydrochloride-plus kit (Zymed) was utilized according to the manufacturer's suggestions. Immunoreactive sections were briefly counterstained (15 sec) with hematoxylin solution Gill no. 3 (Sigma). Sections were examined at 20× and 40× magnification using a Zeiss microscope. Images were captured using a SPOT camera and software (Diagnostic Systems, Sterling Heights, MI). Final images were generated using Photoshop 5.5 (Adobe Systems, San Jose, CA). Experiments for each size follicle and different stages of CL were repeated three times and with at least three different pigs. At least 15 animals were evaluated during this study. For several tissue blocks, two or more sections were probed using identical conditions to confirm the reproducibility of results. Nuclei were scored as positive when a brown color was present [30]. The percentage of positive cell nuclei was estimated by counting 100 cells per structure from at least three different animals.

Because blocking antigens are not commercially available for preabsorbing these GATA-4 and GATA-6 antibodies, we incubated some slides with GATA-4 or GATA-6 antibodies preabsorbed with in vitro-translated porcine GATA-4 or GATA-6, respectively. Preabsorption was performed for 2 h at room temperature using 2 μg antibody/50 μl translation product plus 50 μl D-PBS. The expression vector pcDNA3.1 alone (control) and vectors containing porcine GATA-4 or GATA-6 cDNAs were in vitro translated using the TNT T7 Coupled Wheat Germ Extract System (Promega).

Electrophoretic Mobility Shift Assays

Granulosa cells were aspirated from measured antral follicles of 1–2, 3–4, and 8–10 mm by needle aspiration and scraping the inner lining of the follicle. Luteal tissue was dissected away from the capsule and minced with a razor blade on ice. Red blood cells in each preparation were lysed by briefly resuspending cell pellets in cold sterile water followed by washing with cold D-PBS. Nuclear proteins were then isolated as previously described [27]. Nuclear extracts were dialyzed at 4°C in buffer containing 50 mM Hepes, pH 7.5, 80 mM KCl, 0.1 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.1 mM PMSF, 20% glycerol, 1 mM $NaVO_3$, and 1 mM NaF. Protein concentrations were determined as described above. The GATA oligonucleotide contains two GATA consensus sequences and was labeled using γ-^{32}P-ATP (3000 Ci/mmol) and T4 polynucleotide kinase. Nuclear protein extracts (7 or 10 μg) were incubated with 100 000-cpm oligonucleotide in binding buffer (10 mM Tris, pH 7.5, 0.5 mM DTT, 5% glycerol, 0.5 mM PMSF, 4 ng/ml aprotinin, 2 ng/ml leupeptin, 80 mM KCl, and 2 μg poly dI-dC) for 30 min on ice. For supershift and competition assays, GATA-4 (C-20X) and GATA-6 (N-18X) antibodies (4–6 μg) or cold competitor oligo (100-fold excess) were preincubated 30 min on ice prior to the addition of hot oligonucleotide. Normal goat IgG was used as a negative control. After addition of gel loading buffer (25 mM Tris, pH 7.5, 4% glycerol, and 0.02% bromophenol blue), samples were electrophoresed on 5% native acrylamide gels in 0.5× Tris-borate-EDTA running buffer at 150 V for 3 h. Gels were dried and exposed to BioMax MR film at −70°C with intensifying screens.

Transfections

Porcine granulosa cells were isolated from 1- to 5-mm follicles from immature porcine ovaries by needle aspiration and were cultured in modified Eagle medium (MEM) supplemented with 3% fetal calf serum (FCS) and antibiotics as previously described [23]. Cells were plated at a density of 1.5–1.8 $\times 10^6$ cells/well in 24-well dishes (Falcon, Franklin Lakes, NJ), and the medium was changed after the initial 24 h. After 48 h in culture, cells were rinsed with MEM only and transfected using 6 μl Lipofectamine reagent and 1 μg plasmid DNA per well in MEM for 5 h. Reporter gene plasmids included the porcine inhibin-α subunit promoter and the StAR promoter-luciferase constructs described above or the promoterless luciferase vector pALuc. Cotransfection was performed with 0.1 μg of expression vector containing GATA-4 and GATA-6 cDNAs or with pcDNA3.1 alone plus 0.9 μg of each reporter construct. Duplicate wells were transfected in each experiment. Following transfection, medium was replaced and cells were cultured for another 12 h in MEM supplemented with 3% FCS, followed by an additional 4 h in free-serum MEM. Cells were lysed in 1× Passive Lysis buffer (Promega), and lysate was assayed as previously described [27]. The protein concentrations of the lysates were used to normalize the luciferase values.

Statistical Analyses

Data from RT-PCR studies (GATA mRNA/S16 mRNA) and transfection experiments (light units/μg protein) were subjected to ANOVA. When differences were significant ($P \leq 0.05$), means were analyzed with the Fisher protected least significant difference (PLSD) test. Comparisons were made with Stat View software (SAS Institute, Cary, NC).

RESULTS

The cloning of the porcine GATA-4 coding sequence revealed a cDNA with a high GC nucleotide content (70% overall). The predicted translation of the GATA-4 cDNA is shown in Figure 1. BLAST alignment [24] showed amino acid identities of 92% with human, 90% with rat, and 89% with mouse coding sequences. Porcine GATA-4 exhibited 46% amino acid identity with porcine GATA-6, which increased to 84% amino acid matches in the zinc finger regions.

The presence of GATA-4 and GATA-6 transcripts was confirmed by semiquantitative RT-PCR in granulosa cells, antral follicles of different sizes, CH, and midluteal phase CL. Figure 2, A and B, shows the autoradiographic bands of amplified GATA-4 and S16 cDNAs from one set of tissue, and graphs show a summary of three sets of tissues for GATA-4 message expression. Figure 2, C and D, represent the autoradiographic bands and analysis for GATA-6 in the same structures as in Figure 2, A and B. No statistically significant differences in GATA-4 transcript expression were observed between granulosa cells from antral follicles of 1–2, 3–4, or 8–10 mm. However, GATA-6 message expression was significantly higher ($P \leq 0.05$) in granulosa cells from 8- to 10-mm antral follicles when compared with granulosa cells from 1- to 2-mm or 3- to 4-mm follicles. GATA-4 transcript expression was significantly higher ($P \leq 0.05$) in 8- to 10-mm antral follicles and CH when compared with 1- to 2-mm antral follicles or CL. No significant differences in GATA-6 transcript expression were found by ANOVA among follicles of different sizes, CH, or CL, but 8- to 10-mm follicles tended to be numerically higher. No PCR product was detected when diethyl pyrocarbonate-treated water was used as a template for RT-PCR.

Immunoblot analyses were conducted to examine the cyclic expression of GATA-4 and GATA-6 proteins using

FIG. 5. Immunohistochemical localization of GATA-6 protein in porcine ovarian structures. Positive staining is shown in brown. Ovaries were stained with GATA-6 antibody and secondary antibody. In each slide, granulosa (GC), theca (TC), stromal (St), and luteal (LC) cells and oocytes (Oc) and blood vessels (BV) are indicated. Bars = 20 μm. **A**) Section of ovary including a 1- to 2-mm antral follicle and midluteal phase CL using IgG in place of the primary antibody (control slide). **B**) Same as section **A** but in the presence of the GATA-6 primary antibody. **A** and **B** are 20×. **C**) Section of ovary including several primordial follicles. These follicles were located in a section surrounded by atretic follicles. **D**) Section of ovary including a primary follicle and 3- to 4-mm antral follicle. **E**) Section of ovary including a 7-mm antral follicle. **F**) Section of ovary including a midluteal phase CL. **C**–**F** are 40×.

whole cell extracts from granulosa cells from antral follicles (1–2, 3–4, and 8–10 mm), CH, and midluteal phase CL from porcine ovaries. Two predominant immunoreactive proteins with molecular masses of approximately 53 and 55 kDa for GATA-4 (Fig. 3A and one 55-kDa protein for GATA-6 (Fig. 3B) were detected in all tissues examined. GATA-4 and GATA-6 appear to be strongly expressed in granulosa cells and follicles of different sizes, CH, and midluteal phase CL. We could not detect consistent variations in protein expression by this method. As negative controls, parallel membranes were incubated with normal rabbit IgG in place of the primary antibody and yielded no bands at the molecular masses of GATA-4 or GATA-6 (data not shown). We found no consistent variation in the intensity of the actin band among different tissues.

Two immunoreactive bands of GATA-4 were detected in whole cell extracts of all selected porcine ovarian tissues (Fig. 3A). Because GATA-4 can be phosphorylated [31], the upper band obtained may represent a phosphorylated form and the lower band may represent the nonphosphorylated form of the protein. To test this hypothesis, nuclear extracts from granulosa cells from 1- to 2-mm antral follicles and CL were isolated and treated in the presence or absence of λ-PPase followed by immunoblot analysis. Treatment with this enzyme abolished the upper band and enhanced the lower band of GATA-4 in granulosa cells and CL, indicating the presence of a phosphorylated isoform (Fig. 3C). In this study, the same results were obtained from three different sets of granulosa cells and CL. Similar studies were performed for GATA-6, but no size shift was observed (data not shown).

In previous studies, GATA-4 protein was detected in the nuclei of mouse and human granulosa cells and in lesser amounts in thecal cells [14, 16]. To localize GATA-4 and GATA-6 proteins in porcine ovaries, immunohistochemistry was conducted on different sections of porcine ovaries containing primordial, primary, and antral follicles, CH, midluteal phase CL, or regressive CL. Immunohistochemical localization showed that 90%–95% of granulosa cells of healthy primordial, unilaminar, multilaminar, and antral follicles of different sizes exhibited nuclear staining for both GATA-4 (Fig. 4, A–F) and GATA-6 (Fig. 5, A–F). Strong immunoreactivity was observed in both cumulus and mural granulosa cells of antral follicles (data not shown). The number of immunopositive thecal cells was dependent upon the stage of follicular maturation, with 20%–50% of cells exhibiting nuclear staining in multilaminar primary follicles, whereas 50%–70% of thecal cells were positive in antral follicles. In CH and healthy CL, >90% of luteal cells displayed nuclear immunoreactivity for both GATA-4 (Fig. 4, E and F) and GATA-6 (Fig. 5, E and F). Regressing CL showed a decrease in GATA-immunopositive cells (40%–50%). In regressing CL, positive luteal cells for GATA-4 and GATA-6 appeared to be clustered and were not uniformly distributed through the tissue (data not shown).

Control staining of sections using normal rabbit IgG in place of the primary antibody produced only weak cytoplasmic staining of granulosa and thecal cells and oocytes (Figs. 4A and 5A). Moderately intense oocyte staining with GATA-4 antibodies appeared to increase with follicular maturation, with an increase from 50% of oocytes in primordial and primary follicles to 100% in antral follicles. Oocyte staining of GATA-6 did not exhibit cyclic variations, and 100% of the oocytes stained strongly positive. A few sparse stromal cells were immunopositive for both GATA factors, with more cells staining for GATA-6. In atretic antral follicles, only the granulosa cells closest to the basement membrane stained for GATA-4 (Fig. 6F). In these atretic follicles, GATA-6 was localized to both granulosa cells residing on the basement membrane and detached apoptotic granulosa cells and thecal cells (Fig. 6G). No significant staining was detected in germinal epithelium (data not shown). Preabsorption of the GATA-4 antibody with in vitro-translated porcine GATA-4 abolished all nuclear immunoreactivity, and preabsorption of the GATA-6 antibody with porcine GATA-6 eliminated the majority of nuclear immunoreactivity (data not shown).

GATA-4 and GATA-6 have been previously localized to blood vessels [8, 10, 32, 33]. Therefore, we also evaluated the localization of GATA-4 and GATA-6 factors in blood vessels surrounding porcine ovarian follicles, CH, and CL (Fig. 6, A–D). Immunoreactivity for GATA-4 and GATA-6 was present in most blood vessels. The nuclei of endothelial cells (65%–100%) were immunopositive for both factors. The abundance of positive vascular smooth muscle cell nuclei was highly variable (10%–70%). Control staining of sections with normal rabbit IgG produced only weak cytoplasmic staining of smooth muscle cells.

Because GATA-4 and GATA-6 proteins can bind to the DNA consensus sequence (A/T)GATA(A/G) in target gene promoters [13, 34], electrophoretic mobility shift assays were performed using select tissues to determine the binding capacities of GATA-4 and GATA-6. Figure 7 shows a representative autoradiogram of nuclear protein binding to GATA consensus oligonucleotide. The GATA oligonucleotide bound nuclear protein from granulosa cells from antral follicles (1–2, 3–4, and 8–10 mm) and CL. However, the binding was consistently higher in granulosa cells isolated from 8- to 10-mm preovulatory follicles. When polyclonal antibodies specific for GATA-4 or GATA-6 protein were added to each reaction, supershifts were produced in all tissues. Supershifts were strongest in nuclear extracts derived from granulosa cells from 8- to 10-mm (preovulatory) follicles. The multiple bands may reflect binding of one or two molecules of GATA-4 and/or GATA-6 because the oligonucleotide contains two GATA sites. In this study, three or four different preparations of each nuclear extract were evaluated, and qualitatively similar results were obtained. GATA-4 binding appeared to predominate in these tissues.

Previous studies have shown that GATA-4 can transactivate several steroidogenic and developmental gene promoters in gonadal and nongonadal cell lines [13, 18, 34]. The StAR and inhibin-α genes are both expressed in differentiating granulosa cells [35–39]. Therefore, we tested the ability of our porcine GATA-4 and GATA-6 expression constructs to transactivate the porcine StAR and inhibin-α subunit promoter luciferase constructs in primary cultures of luteinizing granulosa cells (Fig. 8). GATA-4 and GATA-6 significantly increased ($P \leq 0.05$) porcine StAR promoter-driven luciferase activity by 6.6-fold ± 1.9-fold and 9.8-fold ± 3.9-fold, respectively, compared with the empty expression vector (pcDNA3.1) in granulosa cells. When transfected with the inhibin-α promoter construct, GATA-4 and GATA-6 cotransfected cultures tended to be higher than control by 1.5-fold ± 0.27-fold and 2.1-fold ± 0.32-fold, respectively, but this increase was not significant. However, basal expression of the inhibin-α construct was on average 3-fold higher than that for the StAR construct. Transfections with the pALuc vector alone gave values close to the threshold of detection (data not shown).

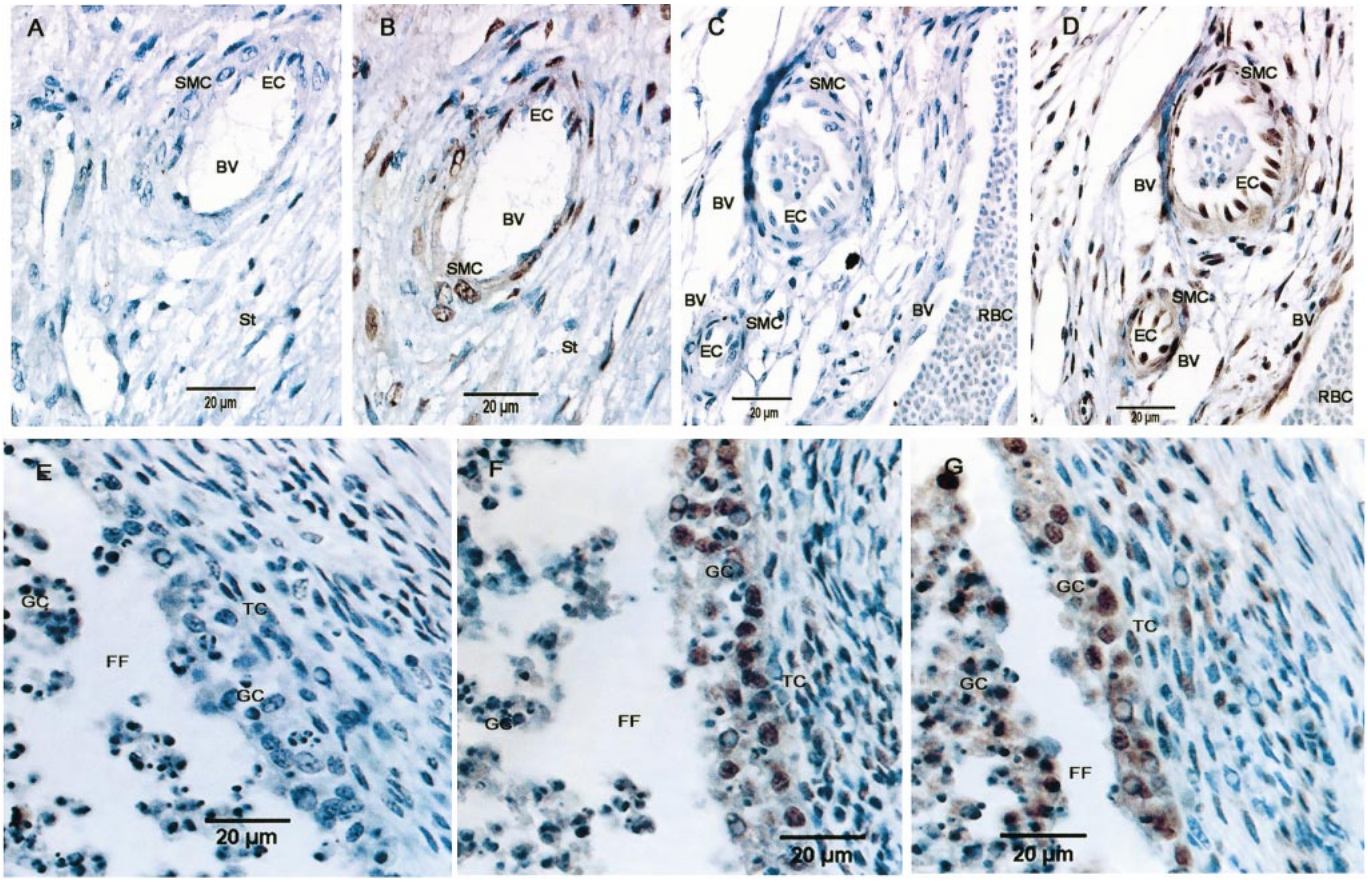


FIG. 6. Immunohistochemical localization of GATA-4 and GATA-6 protein in porcine ovarian blood vessels and atretic antral follicles. Positive staining is shown in brown. Ovarian blood vessels and atretic antral follicles were stained with GATA-4 or GATA-6 antibody and secondary antibody. In each slide, endothelial (EC), smooth muscle (SMC), granulosa (GC), theca (TC), stromal (St), and red blood (RBC) cells, blood vessels (BV), and follicular fluid (FF) are indicated. Bars = 20 μm. Top panel represents the immunohistochemical localization of GATA-4 and GATA-6 protein in ovarian BV associated with CL or CH. **A**) Section of ovary including a BV. IgG was used in place of the primary antibody (control slide). **B**) Same as section **A** but in the presence of the GATA-4 primary antibody. **C**) Section of ovary including BV. IgG was used in place of the primary antibody (control slide). **D**) Same as section **C** but in the presence of the GATA-6 primary antibody. Bottom panel represents the immunohistochemical localization of GATA-4 and GATA-6 protein in an atretic antral follicle. **E**) Section of ovary including an atretic antral follicle. IgG was used in place of the primary antibody (control slide). **F**) Same as section **E** but in the presence of the GATA-4 primary antibody. **G**) Same as section **E** but in the presence of the GATA-6 primary antibody. Magnification ×40.

DISCUSSION

In this study, we demonstrated the specific expression and localization of the transcription factors GATA-4 and GATA-6 in the ovaries of spontaneously cycling pigs. In concordance with studies conducted in humans and mice, we found the expression of GATA-4 and GATA-6 mRNAs in all follicular structures. GATA-4 mRNA increased in 8- to 10-mm follicles (preovulatory follicles) and then decreased after ovulation; levels in CL were similar to those in 1- to 4-mm follicles. No significant differences in GATA-4 mRNA were found among the granulosa cells of antral follicles of different sizes. Therefore, the changes in the expression of GATA-4 mRNA in preovulatory follicles may be due to the presence of transcripts in thecal cells, oocytes, and even in follicle-associated vascular cells; our immunohistochemical studies showed that GATA-4 and GATA-6 were present in these structures. GATA-6 mRNA was also detected in all follicles and luteal structures; however, GATA-6 mRNA was significantly increased in granulosa cells only during the preovulatory stage. No significant differences in GATA-6 mRNA were found between follicles from different stages of maturation. These results could indicate that the majority of GATA-6 mRNA in follicles before ovulation could be produced by differentiating granulosa cells.

In mice and humans, GATA-4 proteins are expressed in granulosa cells before ovulation [14, 16]. However, at the time of these previous studies GATA-6 antibodies were not available and thus there is no information about GATA-6 protein expression. As shown by immunoblot analyses and immunohistochemistry, porcine GATA-4 and GATA-6 proteins were expressed in all follicles and postovulatory structures. No dramatic changes in the abundance of GATA-4 or GATA-6 proteins in follicles or luteal structures were observed.

Two isoforms of GATA-4 protein (approximately 53 and 55 kDa) were detected by immunoblot analysis in whole cell extracts from porcine ovarian structures. However, the 55-kDa isoform was predominant in nuclear protein extracts. These results, together with λ-phosphatase treatment experiments, indicate that the phosphorylated form predominates in the nucleus. The significance of this observation in ovary is uncertain at this time, but GATA-4 phosphorylation promotes transactivation ability in cardiovascular cells [31, 40, 41]. GATA-4 activity may be regulated by posttranslational events such as phosphorylation rather than by protein abundance.

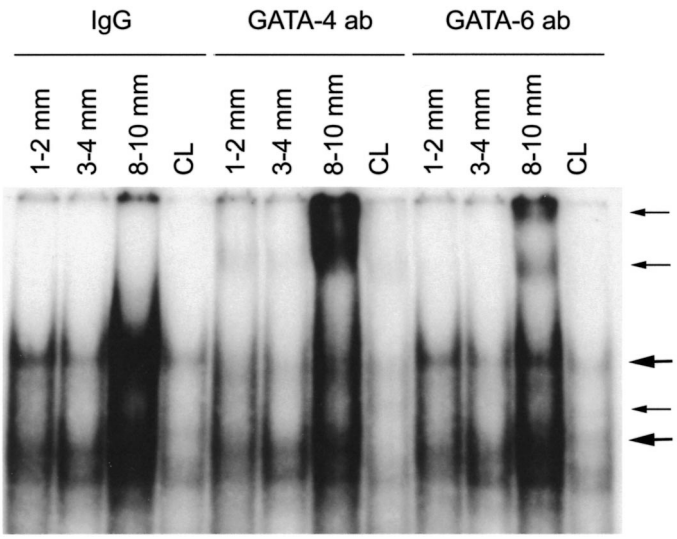


FIG. 7. DNA-binding abilities of GATA-4 and GATA-6 in granulosa cell nuclear protein extracts (7 μg) from antral follicles 1–2, 3–4, and 8–10 mm and from midluteal phase CL as assessed by electrophoretic mobility shift assay. The thick arrows indicate the GATA-4 and/or GATA-6 protein-DNA complexes, and the thin arrows indicate supershifts. Similar results were obtained from three or four different samples of each tissue.

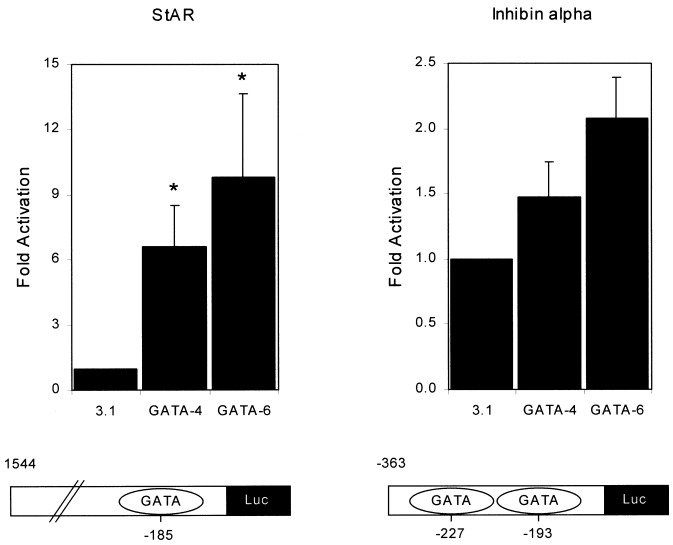


FIG. 8. Transactivation abilities of porcine GATA-4 and GATA-6 cDNAs on StAR and inhibin-α subunit gene promoters. GATA-4 and GATA-6 expression vectors were cotransfected with either −1544-bp StAR or −363-bp inhibin-α promoter-luciferase constructs. Luciferase activity (light units/μg protein) was normalized to the empty pcDNA3.1 expression vector (3.1) control in each experiment. Data represent the mean fold activation relative to control ± SEM for five independent experiments. Asterisks indicate values significantly different from control vector (3.1) as determined by ANOVA and Fisher PLSD test ($P \leq 0.05$).

Immunohistochemical analyses conducted in this study provided the first localization of both GATA-4 and GATA-6 immunoreactive proteins in spontaneously cycling pigs. GATA-4 protein is primarily localized to the nucleus in mouse and human ovarian cells and mouse testicular cells [13, 14, 16]. During this study, GATA-4 and GATA-6 proteins were predominantly located in the nuclei of the granulosa, thecal cells, and luteal cells of CH and CL. Cytoplasmic staining was more pronounced with GATA-6 than with GATA-4. Our results indicate that the temporal localization in pigs is slightly different than that in mice; GATA-4 is barely detectable in murine CL [14], whereas luteal expression of GATA-4 and GATA-6 in pigs decreased only during CL regression.

GATA-4 and GATA-6 were also present in porcine ovarian blood vessels. GATA-6 regulates vascular smooth muscle cell differentiation [8, 10, 32, 42], and GATA-4 is present in developing heart [9, 43, 44]. GATA-4 regulates expression of a number of cardiac genes, such as α-myosin heavy chain, atrial natriuretic factor, brain natriuretic peptide, and the sodium/calcium exchanger [45–48]. In addition, GATA-4 was implicated as a regulator of angiotensin II type-1A receptor promoter in response to pressure overload stimulation in the adult rat heart [49]. Therefore, in ovary, GATA-4 and GATA-6 may participate in the development and maintenance of ovarian blood vessels. Alternatively, extrapolating from their role in the cardiovascular system, these factors could be involved in management or responses to increases in intrafollicular pressure during the preovulatory period. These speculations warrant further study.

In both mouse and human granulosa cells, GATA-4 expression is correlated with active cell proliferation and low levels of apoptosis [14]. GATA-4 mRNA levels decreased abruptly in murine granulosa cells before follicular atresia through programmed cell death [14], indirectly implying that this transcription factor may regulate apoptosis. Likewise, in cardiac muscle cells GATA-4 has been implicated as a survival factor [50, 51], and downregulation of GATA-1 and GATA-2 have also been associated with increased apoptosis in hematopoietic and other nonovarian cells [4, 52]. Our immunohistochemical data indicate that GATA-4 decreases in all granulosa cells of atretic antral follicles. Therefore, in porcine ovary, GATA-4 expression could be involved in survival of granulosa and thecal cells of the growing follicles and possibly endothelial and vascular smooth muscle cells.

GATA-4 and GATA-6 DNA binding (shown by electrophoretic mobility shift assay) was increased in the granulosa cells from 8- to 10-mm follicles. These data implicate an increased role for these factors during the periovulatory period. This period is associated in rats and primates with a decline in granulosa cell mitosis and a transient increase in cyclin-dependent kinase inhibitor $p21^{Cip1}$ [53, 54]. GATA-6 upregulates cyclin-dependent kinase inhibitor $p21^{Cip}$ during vascular smooth muscle cell differentiation [55]. More studies are needed to determine whether GATA-4 and GATA-6 control cell cycle arrest and differentiation events in ovarian follicular or vascular cells.

Our transfection data implicate both GATA-4 and GATA-6 in the activation of the StAR gene. StAR mediates the transfer of cholesterol from the outer to the inner mitochondrial membrane, where it is converted to pregnenolone [56]. StAR is undetectable in granulosa cells of follicles prior to the LH surge but is upregulated after ovulation and is expressed in cultured luteinizing granulosa cells [35, 36, 57]. Our study shows that GATA-4 overexpression can regulate the StAR gene in primary ovarian cells, consistent with previous observations for GATA-4 in clonal cells [20]. We also observed that GATA-6 can regulate the StAR gene in primary ovarian cells. Because both GATA factors increased expression of the StAR gene and both GATA-4 and GATA-6 protein levels are high throughout the cycle, these factors must cooperate with other cell-specific transcription factors to determine cycle-specific expression. StAR gene expression involves transcription factors SF-1 and CEBP [58, 59]. In immature Sertoli cells, GATA-4 physically in-

teracts with SF-1, enhancing transcriptional synergy on the Müllerian inhibiting substance gene promoter [60]. In a recent study in rat postnatal cardiomyocytes, GATA-4 and GATA-6 interacted to regulate atrial natriuretic factor and B-type natriuretic peptide gene expression [61]. GATA-4 interacts with the transcriptional modifying protein, FOG-2, resulting in transcriptional activation or repression in a gene-specific manner [62, 63]. In addition, GATA-6 can interact with coactivator p300 in vascular smooth cells [64]. Whether GATA-4 or GATA-6 directly interact with each other or other transcription factors will be addressed in future studies.

GATA-4 or GATA-6 did not significantly stimulate the expression of the porcine inhibin-α subunit gene promoter construct. Inhibin-α is naturally expressed by maturing granulosa cells and luteal cells in vivo and by cultured luteinizing granulosa cells [38, 39, 65–67]. The mean expression of the porcine inhibin-α gene promoter construct in transfected luteinizing granulosa cells with either GATA cDNA was on average 1.5- to 2.1-fold higher than that for the empty expression vector. In contrast, Tremblay and Viger [18] showed that GATA-4 stimulated the murine inhibin-α gene promoter in transfected monkey kidney CV-1 cells by approximately 6-fold. The discrepant results of these two studies could be due to the fact that granulosa cells themselves have endogenous levels of GATA-4 and GATA-6 and exhibited high inhibin-α basal activity. The inhibin-α gene promoter contains two GATA consensus sequences, which may account for its higher basal activity compared with the StAR construct, which only contains one GATA site.

Or results indicate that GATA-4 and GATA-6 transcripts and protein are present before and after ovulation in porcine ovaries and can activate expression of at least one ovarian gene promoter, StAR, in primary ovarian cells. Expression of both factors may be critical just before ovulation because an increase in GATA DNA binding was observed in granulosa cells nuclear extracts from preovulatory follicles. Given that GATA-4 and GATA-6 have highly conserved DNA binding and transactivation domains [2, 68], their presence in the same ovarian cells suggests a possible redundancy for these factors in ovarian function. Further functional studies are needed to clarify the roles of porcine GATA-4 and GATA-6 in ovarian follicular development, ovulation, and luteal function and the ovarian vascular network.

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