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Expression and Hormonal Regulation of Transcription Factors GATA-4 and GATA-6 in the Mouse Ovary*

MARKKU HEIKINHEIMO†, MARINA ERMOLAEVA†, MALGORZATA BIELINSKA, NAFIS A. RAHMAN, NAOKO NARITA, ILPO T. HUHTANIEMI, JUHA S. TAPANAINEN, AND DAVID B. WILSON‡

Children's Hospital (M.H.), University of Helsinki, 00290 Helsinki, Finland; Departments of Pediatrics (M.H., M.E., M.B., N.N., D.B.W.) and Molecular Biology and Pharmacology (D.B.W.), Washington University, St. Louis, Missouri 63110; Department of Obstetrics and Gynecology (J.S.T.), University of Oulu, 90220 Oulu, Finland; Department of Physiology (N.A.R., I.T.H.), University of Turku, 20520 Turku, Finland

ABSTRACT

Two members of the GATA-binding family of transcription factors, GATA-4 and GATA-6, are expressed in the vertebrate ovary. To gain insight into the role of these factors in ovarian cell differentiation and function, we used *in situ* hybridization to determine the patterns of expression of GATA-4 and GATA-6 in mouse ovary during development and in response to hormonal stimulation. GATA-4 messenger RNA (mRNA) was first evident in the ovary around the time of birth. In the adult ovary, abundant GATA-4 mRNA was detected in granulosa cells of primary and antral follicles, with lesser amounts of GATA-4 message detected in theca cells, germinal epithelium, and interstitial cells. Little or no GATA-4 mRNA was found in corpus luteum. GATA-6 message exhibited a different distribution in the ovary, with abundant expression evident in both granulosa cells and corpora lutea. Stimulation of 3-week-old females with PMSG or estrogen enhanced follicular expression of GATA-4 and GATA-6 transcripts. Subsequent induction of ovulation with human CG resulted in a decrease in GATA-4 mRNA expression in granulosa cells, whereas GATA-6 mRNA expression persisted in granulosa cells after ovulation and in corpora lutea. Moreover, follicular apoptosis was associated with a decrease in the expression of GATA-4 but not GATA-6 message. Stimulation of cultured gonadal cell lines with FSH resulted in increased expression of GATA-4 message, whereas GATA-6 mRNA expression was not affected. In light of these findings, the established role of other GATA-binding proteins in hematopoetic cell differentiation and apoptosis, and the presence of conserved GATA motifs in the promoters of genes expressed selectively in ovary, we propose that GATA-4 and GATA-6 play distinct roles in follicular development and luteinization. (*Endocrinology* **138:** 3505–3514, 1997)

THE MOUSE OVARY consists of germ cells and stroma cells, including granulosa cells and theca cells, embedded within a network of interstitial cells. Ovarian development in the mouse begins with the migration of primordial germ cells along the wall of the hindgut to the genital ridge at 10.5–12 days *post coitum* (*p.c.*) (1). Upon arrival in the genital ridge, the germ cells become closely associated with somatic cells, the supporting and interstitial cells from the mesonephric region of the urogenital ridge (2, 3). Epithelial follicular cells (pregranulosa cells) originate from the germinal epithelium and subsequently encircle the germ cells. By 2–3 weeks of life, most of the ovarian cortex is occupied by primary follicles, consisting of oocytes surrounded by a single layer of granulosa cells, a basement membrane, and theca cells (3). Some follicles subsequently enter a growth phase, marked by granulosa cell proliferation, cavitation of the granulosa cell layer, and formation of a fluid-filled antral follicle, and the later phases of this development (from early antral stage) are strictly gonadotropin dependent (4). However, the vast majority of proliferating follicles become atretic via the mechanism of apoptosis (5–8). A small percentage of surviving late stage (Graafian) follicles undergo the process of ovulation, after which the granulosa and theca cells become nonmitotic and form a corpus luteum (9).

Although significant advances have been made in our understanding of ovarian development and function, the transcription factors that determine lineage commitment and cell proliferation in the ovary are not fully understood (10). Among the transcription factors that have recently emerged as potential regulators of gonadal gene expression and function are the GATA-binding proteins, a family of structurally related zinc finger proteins that recognize the consensus sequence (A/T)GATA(A/G), known as the "GATA" motif, which is an essential *cis*-element in the promoters or enhancers of a variety of genes (11). In *Drosophila*, a GATA-binding protein known as dGATAb is expressed in ovarian follicular cells, where it binds and activates the yolk protein genes *Yp1* and *Yp2* (12). In vertebrates, six GATA-binding proteins, termed GATA-1, -2, -3, -4, -5, and -6, have been described (11, 13). The DNA-binding specificities of different members of the vertebrate GATA-binding protein family are largely in-

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Address all correspondence and requests for reprints to: Dr. David B. Wilson, Department of Pediatrics, Box 8116, Washington University School of Medicine, St. Louis Children's Hospital, 1 Children's Place, St. Louis, Missouri 63110. E-mail:wilson_d@kidsa1.wustl.edu.

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† The first two authors contributed equally to this work.

‡ Established Investigator of the AHA.

distinguishable (14, 15), but these transcription factors exhibit different spatial and temporal expression patterns and are therefore presumed to serve different functions in the organism. Through targeted mutagenesis, several of these vertebrate factors have been shown to be critical regulators of differentiation (16–20). For example, GATA-1, -2, and -3, which are expressed in bone marrow cells, are required for normal hematopoiesis (16–18, 20). Moreover, a reduction in GATA-1 expression or activity has been associated with increased apoptosis in erythroid cells (21–23). Northern analysis and RT-PCR assays have shown that two vertebrate GATA-binding proteins, GATA-4 and GATA-6, are expressed in adult ovarian tissue and a limited number of other tissues, including heart, gut epithelium, and yolk sac endoderm (13, 19, 24–31). The cell types within the ovary that express these transcription factors have not been elucidated. Given the established role of GATA-binding proteins in the regulation of gene expression, differentiation, and apoptosis in different cell types, it is intriguing to consider the possibility that transcription factors GATA-4 and GATA-6 participate in the development and/or function of the mammalian ovary. Additional support for the notion that GATA-binding proteins are involved in gonadal development comes from studies of GATA-1, which has been shown to be expressed in a developmental- and stage-specific manner in Sertoli cells of the testes (32, 33).

To gain insight into the role(s) of GATA-4 and GATA-6 in ovarian cell differentiation and function, we have examined the expression of these factors in the mouse ovary during fetal and postnatal development, using *in situ* hybridization. Furthermore, we have determined the temporal and spatial expression of GATA-4 and GATA-6 transcripts in immature mice treated with hormones to induce synchronized follicular development and ovulation. Herein we demonstrate that GATA-4 and GATA-6 have distinct patterns of expression during development and in response to hormonal stimulation.

Materials and Methods

Mouse stocks

Except where indicated, ovaries were obtained from female B6SJLF1/J mice (Jackson Labs, Bar Harbor, ME). Mouse embryos and young neonatal mice were obtained by mating male and female B6SJLF1/J mice. For estimating the embryonal age, noon of the day on which the copulation plug was found was considered as 0.5 days *p.c.*. Precise staging of dissected embryos was performed using *The Atlas of Mouse Development* (34). For animals older than 15 days *p.c.*, sex was assigned on the basis of microscopic morphology.

In situ hybridization

Mouse embryos or dissected tissue were washed briefly in PBS and then frozen in OCT cryopreservation solution (TissueTek, Miles, Inc., Elkhart, IN). Frozen sections (10 μm) were fixed in 4% paraformaldehyde in PBS and subjected to *in situ* hybridization as described (35). Tissue sections were incubated with 1×10^6 cpm of [^{33}P]-labeled antisense or sense riboprobe in a total volume of 80 μl. Antisense and sense riboprobes against the 5′ end of mouse GATA-4 were prepared as described elsewhere (24, 31). To generate antisense riboprobes for GATA-6, a plasmid containing a partial length complementary DNA (cDNA) encoding mouse GATA-6 (30) was linearized with either *Eco*RV or *Pst*I and transcribed *in vitro* with Sp6 polymerase in the presence of [^{33}P]UTP (1000–3000 Ci/mmol, Amersham Life Sciences, Arlington Heights, IL); *Eco*RV digestion yielded a 610 nucleotide probe that recognized the distal zinc finger domain and 3′-end of the GATA-6 coding region, whereas *Pst*I produced a 140-nucleotide probe that recognized only the 3′-end of the GATA-6 coding sequence. These two probes yielded identical results with *in situ* hybridization. Sense riboprobe for GATA-6 was generated with T7 polymerase after linearization with *Bam*HI.

Primary cultures of mouse granulosa cells

Mouse granulosa cells were obtained by follicular puncture using a fine needle as described (36) from 3-week-old immature mice primed with diethylstilbesterol (DES) 12 μg/day ip for 5 days. The cells were cultured on plastic dishes in DMEM supplemented with 10% FCS, L-glutamine (2 mM) and penicillin (100 U/ml), streptomycin (100 μg/ml), and used for immunohistochemistry after 2–3 days in culture.

Immunohistochemistry

Cultured granulosa cells were fixed with 4% paraformaldehyde and subjected to immunohistochemistry using either affinity purified rabbit antimouse GATA-4 IgG (1 μg/ml) (24, 31) or nonimmune IgG as the primary antibody. A commercially available avidin-biotin immunoperoxidase system was employed to visualize bound antibody (Vectastain Elite ABC Kit). 3,3′-diaminobenzidine tetrahydrochloride dihydrate (Sigma Chemical Co., St. Louis, MO) was used as the chromogen and the development reaction occurred in the presence of 0.01% H_2O_2 and 0.03% $NiCl_2$ (37).

Hormonal stimulation of immature mice

Immature female mice, aged 19–21 days, were primed with a single ip injection of 5 U PMSG. Some of these animals were injected with 5 IU human CG (hCG) 48 h later. Mice were killed 48 h after PMSG or 5 h, 16 h, or 5 days after hCG injection to obtain ovaries containing preovulatory, postovulatory, and luteinized follicles (38). In the postovulatory group, ovulation was documented by microscopic demonstration of oocytes in the oviduct. Control animals of the same age did not receive any hormone injections. Each treatment group consisted of 6–9 mice.

Alternatively, immature mice (19–23 days) were treated with steroid hormones, using a modification of a procedure developed for rats (7). Stocks of DES or testosterone were prepared by suspending the steroids at a concentration of 2.5 mg/ml in 95% mineral oil and 5% ethanol. Groups of mice were initially primed by twice daily 0.1 ml ip injections of DES. Two days later, the mice were divided into one of three treatment groups: the first group continued to receive twice daily injections of DES (*i.e.* continued estrogen stimulation), the second group received no further treatment (*i.e.* estrogen withdrawal), and the third group received twice daily 0.1 ml injections of testosterone (*i.e.* estrogen withdrawal plus testosterone treatment). Two days later, the ovaries were harvested and cryosectioned for *in situ* hybridization and apoptosis.

Gonadotropin stimulation of tumor cell lines

MSC-1 Sertoli tumor cells, derived from a transgenic mouse line bearing a human Müllerian inhibiting substance promoter-SV40 T-antigen transgene (39), were obtained from Dr. M. Griswold (University of Washington, Seattle, WA). MSC-1 cells were stably transfected with a cDNA encoding the rat FSH receptor (FSHR) (Eskola, V., M. Savisalo, A. Rannikko, K. Kananen, R. Sprengel, and I. Huhtaniemi, unpublished studies). The resultant cells, termed MSC-1/FSHR, cells were stimulated with recombinant FSH 50 IU/liter for varying lengths of time. The NT-1 granulosa tumor cell line (passage no. 4) was derived from transgenic mice bearing an inhibinα subunit promoter-SV40 T-antigen contruct (40). The cells were stimulated with recombinant FSH (50 IU/liter), hCG (50 μg/liter), or forskolin (10 μM) for the indicated lengths of time. Total RNA was isolated using guanidinium thiocyanate-phenol-chloroform extraction and analyzed for expression of GATA-4 or GATA-6 message using Northern hybridization (24). Twenty micrograms of denatured total RNA was subjected to electrophoresis on a 1% denaturing agarose gel and then transferred onto nylon membranes (Hybond N, Amersham). The membranes were hybridized with [^{32}P]labeled cDNA probes for GATA-4 (24) or GATA-6 (27). Hybridization and washing of the membranes were performed as previously described (24). Hybridization

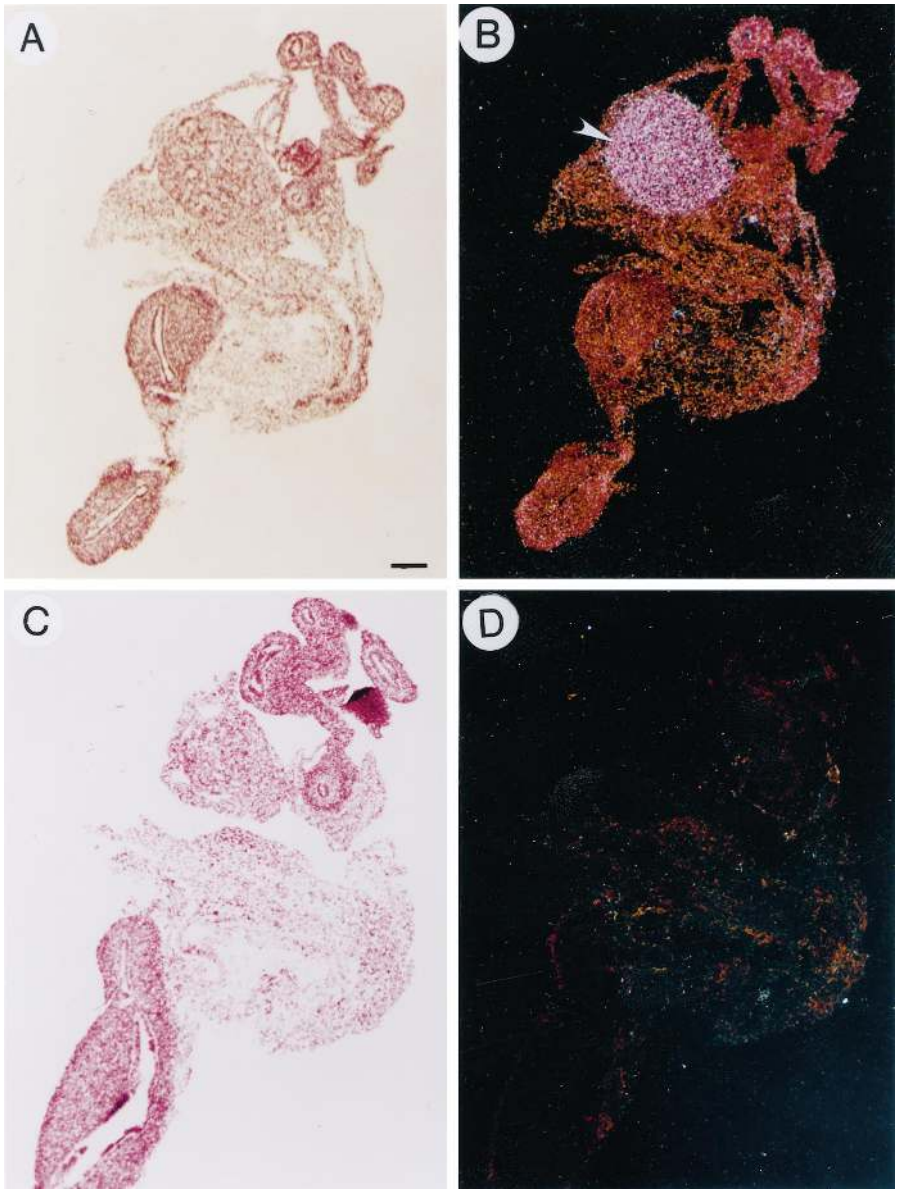

FIG. 1. Expression of GATA-4 and GATA-6 message in newborn mouse ovary. Bright field (A, C) and dark field (B, D) views of sections through adnexal tissue are shown. *In situ* hybridization demonstrates the presence of GATA-4 (B) but not GATA-6 (D) message in the neonatal ovary (*arrow*). *Bar*, 0.2 μm.

signals were detected by autoradiography using Kodak X-Omat AR Diagnostic film XAR5. Autoradiograms were scanned by the Microcomputer Imaging device (MCID, version 1.2, from Imaging Research, Inc., St. Catherines, Ontario, Canada) to quantify messenger RNA (mRNA) species.

In situ apoptosis

Parallel sections of ovaries used for *in situ* hybridization were subjected to *in situ* analysis for apoptosis, using nonisotopic 3′-labeling of DNA in the presence of terminal transferase and digoxigenin-labeled ddUTP (ApopTag Kit, Oncor Inc., Gaithersburg, MD). Labeled DNA was detected by fluorescence conjugated antidigoxigenin antibodies, according to the manufacturer's directions. Sections were lightly counterstained with propidium iodide and photographed using an Olympus fluorescent microscope.

Results

Expression of GATA-4 and GATA-6 during development of the mouse ovary

To refine our understanding of the role of GATA-4 and GATA-6 in ovarian development and function, we used *in situ* hybridization to examine the temporal and spatial distributions of these transcripts in the developing mouse ovary. Similarities and differences between the patterns of expression of GATA-4 and -6 were highlighted by performing *in situ* hybridization for these two transcripts on adjacent tissue sections. As discussed below, we observed cell types that exclusively expressed GATA-4 or GATA-6 message, confirming that there was minimal cross-reactivity between the *in situ* hybridization probes for these two transcription factors. In addition, we performed control *in situ* hybridization experiments with GATA-4 and GATA-6 sense riboprobes, which revealed only background staining (data not shown).

In initial studies, we surveyed fetal mouse tissue sections for expression of GATA-4 or GATA-6 mRNA. Although large amounts of message for GATA-4 and GATA-6 can be detected in heart and intestinal epithelium during fetal development (24, 29–31), only small amounts of these two

transcripts were detected in the fetal ovary between 15 and 18 days *p.c.* (data not shown). Ovarian expression of GATA-4 mRNA increased around the time of birth (Fig. 1, A and B). Expression of GATA-4 by granulosa cells persisted through subsequent stages of development (see below). The onset of GATA-6 mRNA expression in the developing ovary was delayed compared with GATA-4; little GATA-6 message was evident in the ovaries of newborn animals (Fig. 1, C and D), and only trace amounts of GATA-6 mRNA were detectable in granulosa cells of the three week old juvenile ovary (see below).

In the adult ovary, transcripts for both GATA-4 and GATA-6 were readily detected. Large amounts of GATA-4 message were evident in the granulosa cells of primary and antral follicles of mature animals (Fig. 2, C and D). Moderate amounts of GATA-4 expression were also observed in theca cells, the germinal epithelium, and interstitial cells of adult ovaries (Fig. 2, C and D). Expression of GATA-4 mRNA in the theca cell layer was best appreciated under bright field optics, as shown in Fig. 3A. Little or no GATA-4 mRNA expression was seen in oocytes, oviduct, or uterus. As was the case with GATA-4, large amounts of GATA-6 mRNA were evident in granulosa cells of adult animals (Fig. 2, E and F). Moderate levels of GATA-6 message were also detected in the germinal epithelium (Fig. 2E), but no GATA-6 message was evident in theca cells, interstitial cells, or oocytes (Figs. 2, E and F, and 3B). Whereas little GATA-4 mRNA expression was evident in the corpus luteum of adult animals (Fig. 2D), abundant GATA-6 expression was detected in luteal tissue (Fig. 2F). Faint expression of GATA-6 but not GATA-4 message was seen in the proximal oviduct (Fig. 2, C and E). Thus, GATA-4 and GATA-6 display differing patterns of expression in the mouse ovary.

GATA-4 protein is expressed in primary cultures of mouse granulosa cells

To independently verify the *in situ* hybridization findings, primary cultures of mouse granulosa cells were subjected to immunohistochemistry, using an affinity-purified antibody against GATA-4 (24, 31, 41, 42). GATA-4 antigen was detected in the nucleus of the cultured granulosa cells (Fig. 4A), consistent with earlier studies showing that GATA-4 localizes to the nucleus in cardiomyocytes (42, 43) and differentiated F9 embryonal carcinoma cells (41). Control staining of cultured granulosa cells with nonimmune IgG yielded only weak, nonspecific, cytoplasmic staining (Fig. 4B). Thus, both GATA-4 message and protein are present in mouse granulosa cells. Because an antibody directed against GATA-6 was not available, we did not directly assess whether GATA-6 protein is present in granulosa cell cultures.

Expression of GATA-4 and -6 message during follicular maturation, ovulation, and luteinization

To relate GATA-4 and GATA-6 expression to follicular development and luteinization, *in situ* hybridization was

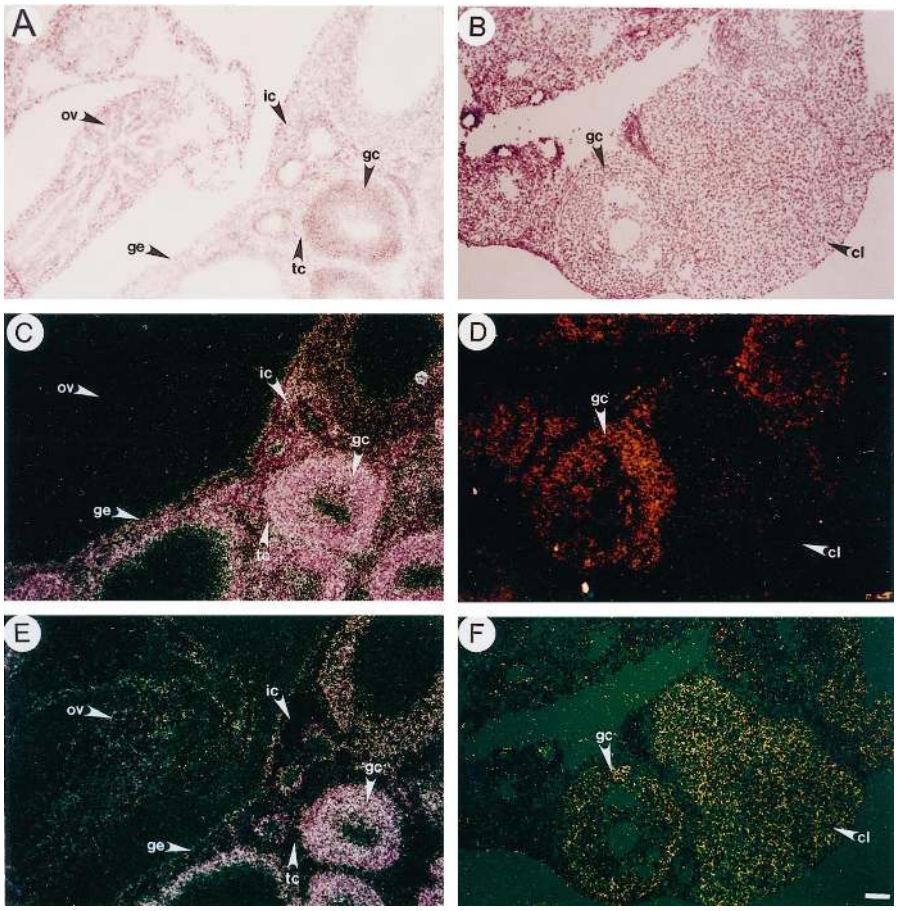

FIG. 2. Expression of GATA-4 and GATA-6 message in adult mouse ovary. Bright field (A, B) and dark field (C–F) views though adult ovary and oviduct are shown. *In situ* hybridization for GATA-4 mRNA (C, D) reveals expression in granulosa cells, theca cells, interstitial cells, and germinal epithelium. *In situ* hybridization for GATA-6 mRNA (E, F) demonstrates expression in granulosa cells, germinal epithelium, corpus luteum, and the oviduct. Abbreviations: cl, corpus luteum; gc, granulosa cells; ge, germinal epithelium; ic, interstitial cells; ov, oviduct; tc, theca cells. *Bar*, 100 μm.

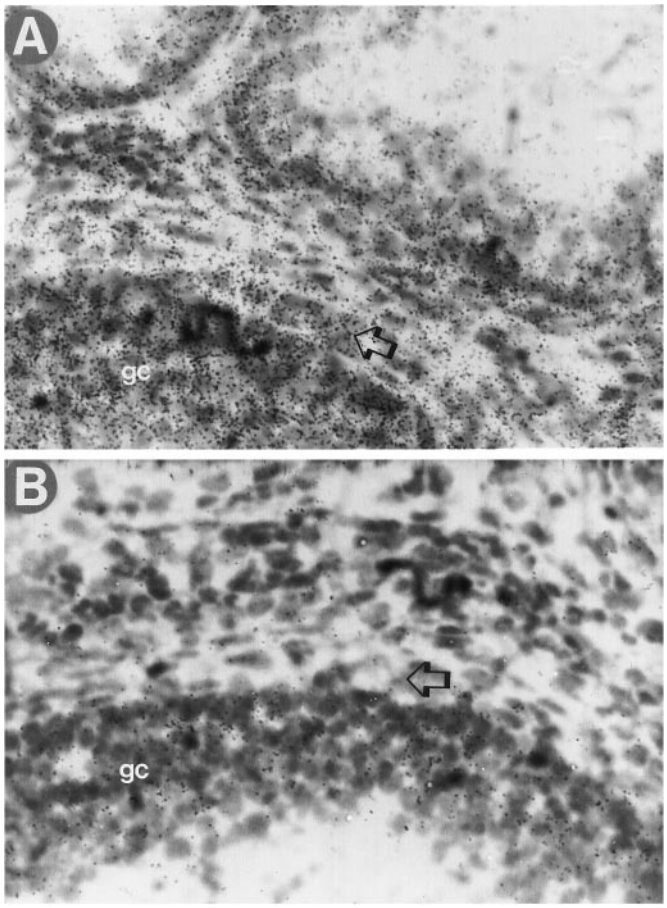

FIG. 3. Expression of GATA-4 and GATA-6 mRNA in theca cells. Adult mouse ovary sections were subjected to *in situ* hybridization for GATA-4 (A) or GATA-6 (B). Bright field views are shown. The *open arrows* point to the theca cell layers surrounding follicles of similar age. Note that GATA-4 but not GATA-6 is expressed in the theca cell layer. gc, Granulosa cells. *Bar*, 30 μm.

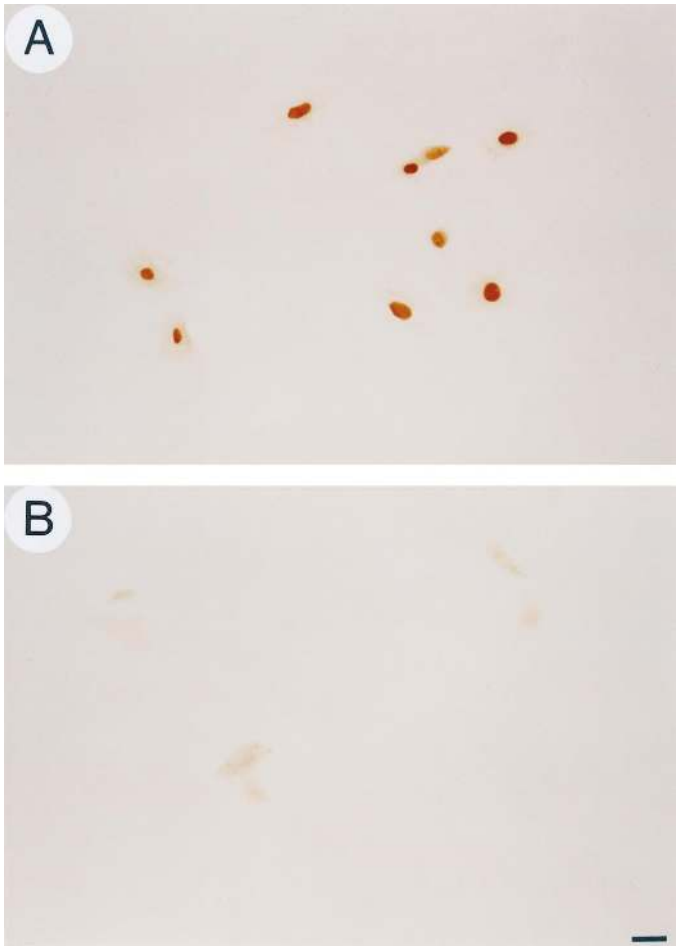

FIG. 4. Immunohistochemistry of GATA-4 in primary cultures of mouse granulosa cells. Granulosa cells were harvested from DES-primed mice and subjected to immunohistochemistry with either (A) affinity purified anti-GATA-4 IgG or (B) nonimmune IgG followed by a peroxidase-conjugated secondary antibody. GATA-4 antigen is evident in the nucleus of the granulosa cells. *Bar*, 20 μm.

performed on immature (3-week-old) mice treated with PMSG followed by hCG to induce synchronized follicular growth and ovulation. High and low magnification views of *in situ* hybridization analysis on PMSG/hCG stimulated ovaries are shown in Figs. 5 and 6, respectively. In unstimulated 3-week-old ovaries, GATA-4 mRNA was evident in granulosa cells of primary and preantral follicles (Fig. 5A), whereas little or no GATA-6 expression was present in these early follicles (Fig. 5B). Robust expression of both GATA-4 and GATA-6 mRNA was seen in the granulosa cells of antral follicles 48 h after PMSG injection (Fig. 5, C and D). Message for GATA-4, but not GATA-6, was also evident in theca cells 48 h after PMSG injection. Administration of hCG to PMSG-primed immature mice resulted in an abrupt decrease in GATA-4 message in granulosa cells of preovulatory follicles, whereas GATA-4 mRNA expression persisted in theca cells (Figs. 5E and 6A). Within preovulatory follicles present at 5 h post hCG administration, cumulus granulosa cells (immediately adjacent to the oocyte) expressed more GATA-4 mRNA than granulosa cells near the follicular basement membrane (Fig. 5E). This finding is consistent with previous studies demonstrating that, at later stages of development, cumulus granulosa cells express different markers and function differently than mural granulosa cells (10). After ovulation, granulosa cells expressed little or no GATA-4 mRNA. When hCG was administered to PMSG-primed immature mice, we observed GATA-6 message in granulosa cells both before and after ovulation (Figs. 5F and 6B). In contrast to GATA-4 mRNA, GATA-6 message in preovulatory follicles was more abundant in mural granulosa cells near the basement membrane than in cumulus granulosa cells. No GATA-6 expression was detected in theca cells in response to hormonal stimulation. Corpora lutea that appeared 5 days after stimulation of PMSG-primed juvenile ovaries with hCG were essentially devoid of GATA-4 message (Figs. 5G and 6C) but expressed significant amounts of GATA-6 (Figs. 5H and 6D). The low magnification views in Fig. 6 emphasize that the hormone-induced changes in expression of GATA-4/6 are uniform.

GATA-4 and GATA-6 expression in cultured gonadal cell lines stimulated with gonadotropins

In addition to the studies on intact ovaries, we measured GATA-4 and GATA-6 mRNA expression in two gonado-

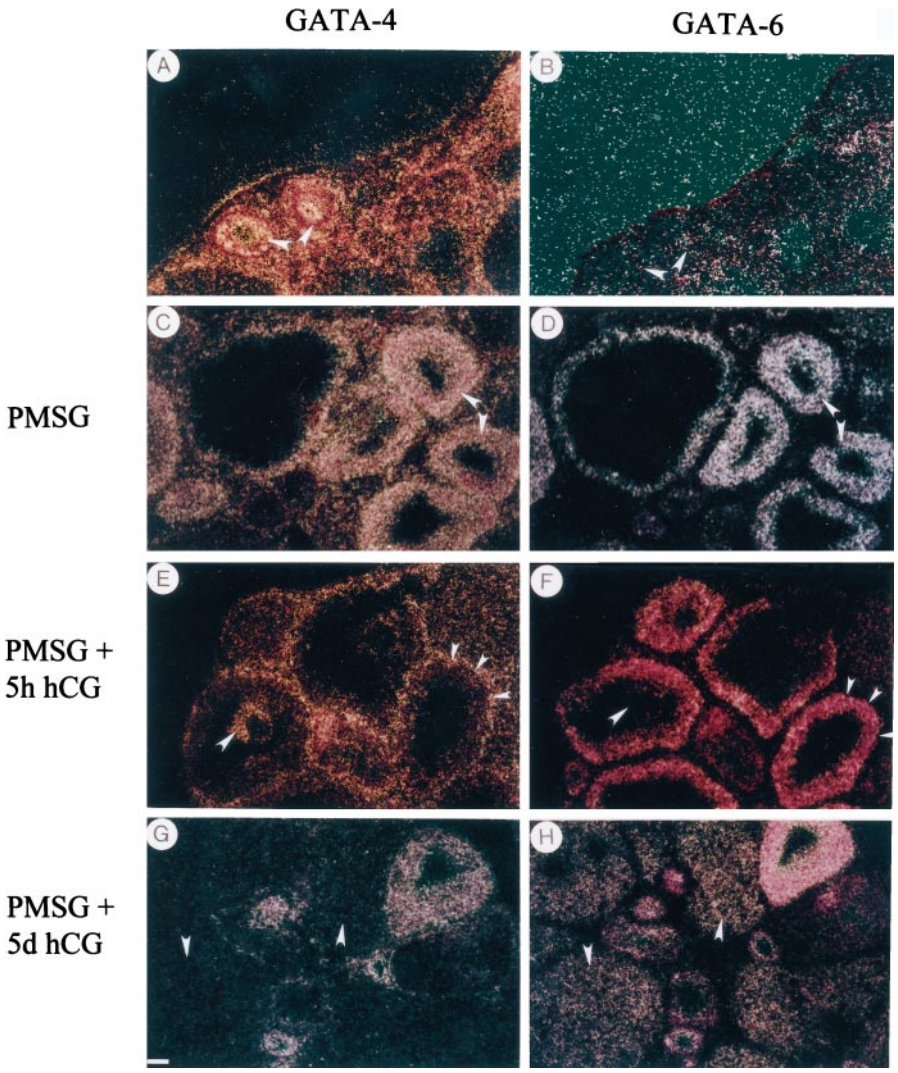

FIG. 5. Expression of GATA-4 and GATA-6 message in gonadotropin-stimulated juvenile ovaries (high magnification views). Three-week-old mice were administered gonadotropins (PMSG ± hCG) to induce follicular maturation and ovulation. At the specified times, ovaries were harvested, sectioned, and subjected to *in situ* hybridization for either GATA-4 (A, C, E, G) or GATA-6 (B, D, F, H) message. In the unstimulated immature ovary, GATA-4 mRNA was evident in the granulosa cells of primordial follicles (A, *arrowheads*), but little GATA-6 message was present at this stage of follicular development (B, *arrowheads*). Forty-eight hours after PMSG administration, we detected abundant expression of both GATA-4 (C, *arrowheads*) and GATA-6 (D, *arrowheads*) transcripts in the granulosa cells of antral follicles. Five hours after administration of hCG to PMSG-primed ovaries, GATA-4 mRNA expression decreased in the granulosa cells of preovulatory follicles (E). This decrease in message was more apparent in granulosa cells near the basement membrane than those near the oocyte (E, *large arrowhead*). Expression of GATA-4 mRNA in theca cells was readily seen at this stage of maturation (E, *small arrowheads*). GATA-6 mRNA expression persisted in the granulosa cells of preovulatory follicles (F). Five days after administration of hCG to PMSG-primed ovaries, little or no GATA-4 message was seen in corpora lutea (G, *arrows*), whereas GATA-6 message was readily detected in luteal glands (H, *arrows*). *Bar*, 100 μm.

tropin-responsive mouse gonadal cell lines. First, we employed mouse MSC-1 Sertoli tumor cells that had been stably transfected with the rat FSH receptor (FSHR) to test whether transcripts encoding GATA-4 and GATA-6 could be induced by FSH treatment. Previous studies have shown that MSC-1/FSHR cells respond to FSH with an increase in cAMP production, whereas nontransfected MSC-1 do not respond to FSH (39). MSC-1/FSHR cells were cultured *in vitro* with recombinant FSH for varying lengths of time, and then GATA-4/-6 expression was determined by Northern analysis (Fig. 7A). FSH stimulation of MSC-1/FSHR cells resulted in an increase in the expression of GATA-4 message, whereas GATA-6 mRNA expression was not affected by FSH treatment. Next, we examined NT-1 cells, a granulosa tumor cell line that exhibits properties of normal granulosa cells, including expression of endogenous gonadotropin receptors, steroidogenic enzymes, and inhibinα (40). We stimulated low passage NT-1 cells with FSH, hCG, or forskolin and measured GATA-4/-6 expression by Northern analysis (Fig. 7B). FSH stimulation resulted in an increase in GATA-4 but not GATA-6 message in NT-1 cells. Forskolin treatment induced expression of both GATA-4 and GATA-6 in these cells. Collectively, these results establish that GATA-4 mRNA can be induced by FSH treatment and show that GATA-4 and GATA-6 are differentially regulated in gonadal cell lines in response to hormone treatment.

Expression of GATA-4 and GATA-6 message during follicular apoptosis

The vast majority of follicles undergo programmed cell death during maturation (5–7, 44). Because decreases in expression or activity of GATA-1 have been associated with apoptosis in erythroid cells (21–23), we explored the relationship between ovarian expression of GATA-4/6 and apoptosis. Adjacent tissue sections of ovary were subjected to *in situ* hybridization (Fig. 8, B and C) and TUNEL (Fig. 8A) assays to examine GATA-4 and GATA-6 expression in follicles undergoing programmed cell death. These studies were carried out on both normal adult ovaries and PMSG/hCG stimulated juvenile ovaries; identical results were obtained in each case. Granulosa cells within large and small apoptotic follicles lacked GATA-4 message (Fig. 8, A and B). In contrast, granulosa cells within these same follicles con-

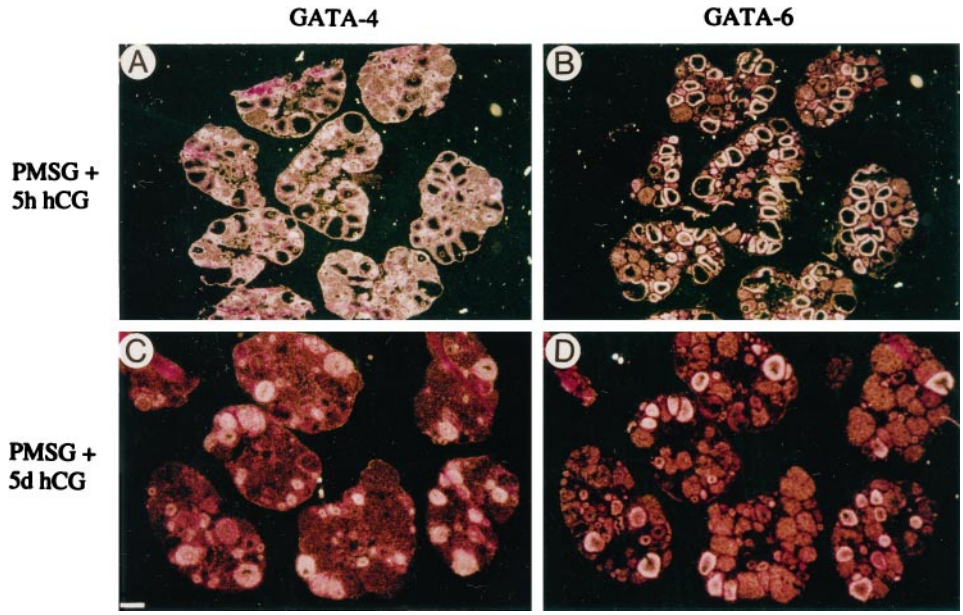

FIG. 6. Expression of GATA-4 and GATA-6 message in gonadotropin-stimulated juvenile ovaries (low magnification views). Three-week-old mice were administered gonadotropins (PMSG ± hCG) to induce follicular maturation and ovulation. At the specified times, ovaries were harvested, sectioned, and subjected to *in situ* hybridization for either GATA-4 (A, C) or GATA-6 (B, D) message. *Bar*, 300 μm.

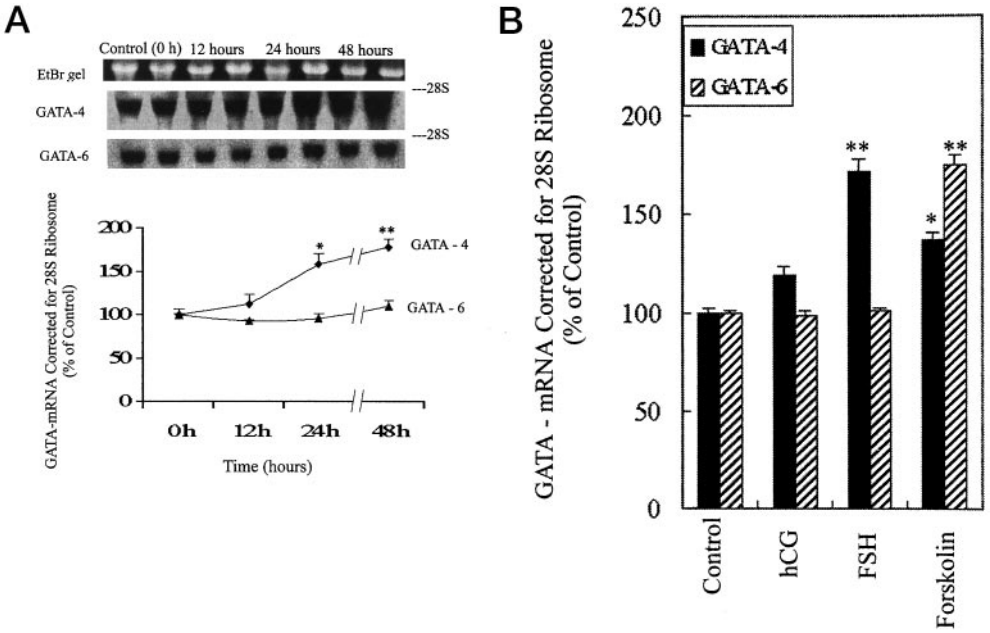

FIG. 7. Expression of GATA-4 and GATA-6 message in hormone-stimulated mouse gonadal tumor cell lines. A, MSC-1/FSHR cells, prepared by stable transfection of MSC-1 Sertoli cells with the FSH receptor, were stimulated with FSH (50 IU/liter) for the indicated lengths of time, and then GATA-4 and GATA-6 mRNA levels were determined by Northern analysis. The first panel shows ethidium bromide staining of a representative RNA gel. The corresponding Northern blots for GATA-4 and GATA-6 mRNA are shown in the second and third panels, respectively. The location of the 28S rRNA band is indicated on the right. The *lower panel* plots the results of densitometric quantification (A.D.U., arbitrary density units), normalized to 28S rRNA levels, for three experiments (mean ± SEM). *, $P < 0.01$ and **, $P < 0.001$ *vs.* the corresponding nonstimulated control group (ANOVA followed by Duncan's new multiple range test). B, NT-1 cells, derived from a granulosa cell tumor, were stimulated with hCG (50 μg/liter), FSH (50 IU/liter), or forskolin (10 μM) for 24 h. GATA-4 and GATA-6 mRNA levels were then determined by Northern analysis. The *bar graph* shows the results of densitometric quantification, normalized to 28S rRNA levels, for three experiments (mean ± SEM). *, $P < 0.01$ and **, $P < 0.001$ *vs.* the corresponding nonstimulated control group (ANOVA followed by Duncan's new multiple range test).

tinued to express significant levels of GATA-6 message (Fig. 8, A and C).

We extended these findings by assessing GATA-4 and GATA-6 expression in granulosa cells undergoing apoptosis in response to estrogen withdrawal ± testosterone administration (7). Immature female mice were stimulated for 2 days with repeated injections of DES. This was followed by one of three treatments: 1) 2 additional days of DES (*i.e.* continued estrogen treatment); 2) no further treatment (*i.e.* estrogen withdrawal); or 3) 2 days of testosterone injections (*i.e.* estrogen withdrawal plus testosterone administration). Ovaries were then harvested and subjected to *in situ* assays

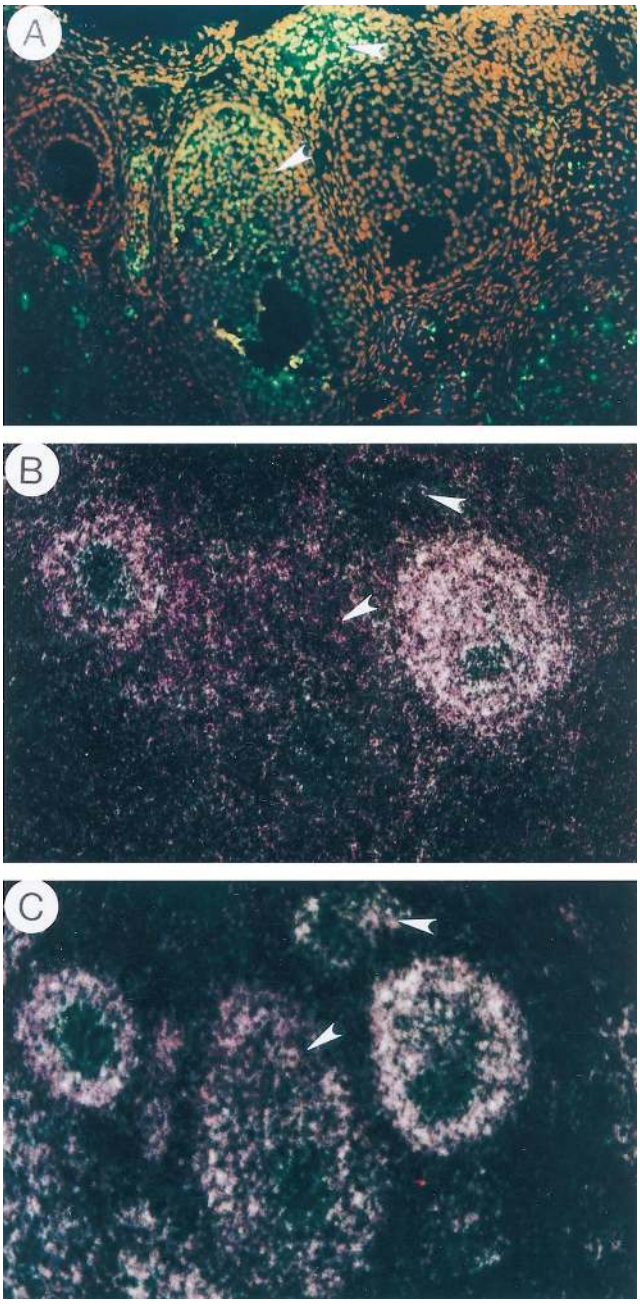

FIG. 8. Expression of GATA-4 and GATA-6 message in apoptotic follicles of the adult mouse. Adjacent tissue sections through adult ovaries were subjected to a fluorescent TUNEL assay for apoptosis (A) or *in situ* hybridization for GATA-4 (B) or GATA-6 (C) message. Granulosa cells in apoptotic follicles, identified by *green* fluorescein isothiocyanate staining, express GATA-6 but not GATA-4 mRNA (*arrowheads*). Nonapoptotic preantral and antral follicles express message for both GATA-4 and GATA-6.

for GATA-4 mRNA, GATA-6 mRNA, and apoptosis. Previous studies have shown that the granulosa cells of estrogen-primed ovaries undergo atresia in response to either estrogen withdrawal or androgen administration (7, 44), although the mechanisms underlying this process remain poorly characterized and may involve other cell types such as theca cells (10). In immature ovaries exposed to DES for 4 days, we observed abundant expression of both GATA-4 (Fig. 9A) and GATA-6 (Fig. 9B) mRNA in granulosa cells. There was also intense expression of GATA-4 message in theca cells and interstitial cells stimulated for 4 days with DES (Fig. 9A). Consistent with earlier observations (7), there were few apoptotic cells in ovaries subjected to continuous estrogen stimulation (Fig. 9C). On the other hand, ovaries subjected to 2 days of DES followed by 2 days of estrogen withdrawal contained increased numbers of apoptotic follicles (Fig. 9F). Little GATA-4 mRNA was detected within the granulosa cells of these apoptotic follicles (Fig. 9D), whereas GATA-6 message was evident in these same follicles (Fig. 9E). That GATA-6 mRNA expression was observed in these apoptotic follicles argues that the down-regulation of GATA-4 message does not merely reflect the end stages of cell death, but rather part of the programmed response to estrogen withdrawal. In DES-primed ovaries treated with testosterone, there were large numbers of severely atretic, TUNEL-positive follicles (Fig. 9I). These follicles had thin granulosa cell layers, likely reflecting the end stages of cell death. These severely atretic follicles expressed neither GATA-4 (Fig. 9G) nor GATA-6 (Fig. 9H) message.

Thus, apoptosis of granulosa cells is associated with an abrupt decrease in GATA-4 expression, whereas GATA-6 expression persists. These findings, together with the developmental expression patterns described above, suggest that GATA-4 message is expressed in potentially mitotic granulosa cells but not in terminally differentiated (*i.e.* luteal) or apoptotic cells, whereas GATA-6 mRNA is present in nonmitotic, terminally differentiated cells and apoptotic cells.

Discussion

We have determined the expression of transcription factors GATA-4 and GATA-6 in the mouse ovary during development and in response to hormone stimulation. GATA-4 message is abundantly expressed in granulosa cells and to a lesser extent in germinal epithelium, theca cells, and interstitial cells of adult ovaries. PMSG or estrogen stimulation of intact, immature ovaries is associated with increased expression of GATA-4 mRNA in granulosa cells. Induction of ovulation in PMSG-primed ovaries with hCG is accompanied by a decrease in GATA-4 message in granulosa cells, and GATA-4 mRNA remains low in regressing follicles and luteal tissue. *In vitro* stimulation of a granulosa and Sertoli tumor cell lines with FSH is also associated with increased expression of GATA-4 message. Follicular atresia via apoptosis is associated with an abrupt decrease in expression of GATA-4. Hence, GATA-4 is expressed in potentially mitotic cells and once these cells become terminally differentiated (from granulosa to luteal cells) or apoptotic, expression is lost. The pattern of GATA-6 mRNA expression in the ovary differs from that of GATA-4. Proliferating granulosa cells express GATA-6 mRNA, but this message is absent from theca cells and interstitial cells. In contrast to GATA-4, induction of ovulation is not associated with decreased expression of GATA-6 in granulosa cells. Moreover, corpus luteum expresses abundant GATA-6 message, and follicular apoptosis is not associated with an abrupt decrease in GATA-6 message. Thus, GATA-6 is expressed in nonmitotic, terminally differentiated cells and in apoptotic cells.

Our findings suggest that GATA-4 and GATA-6 play roles in

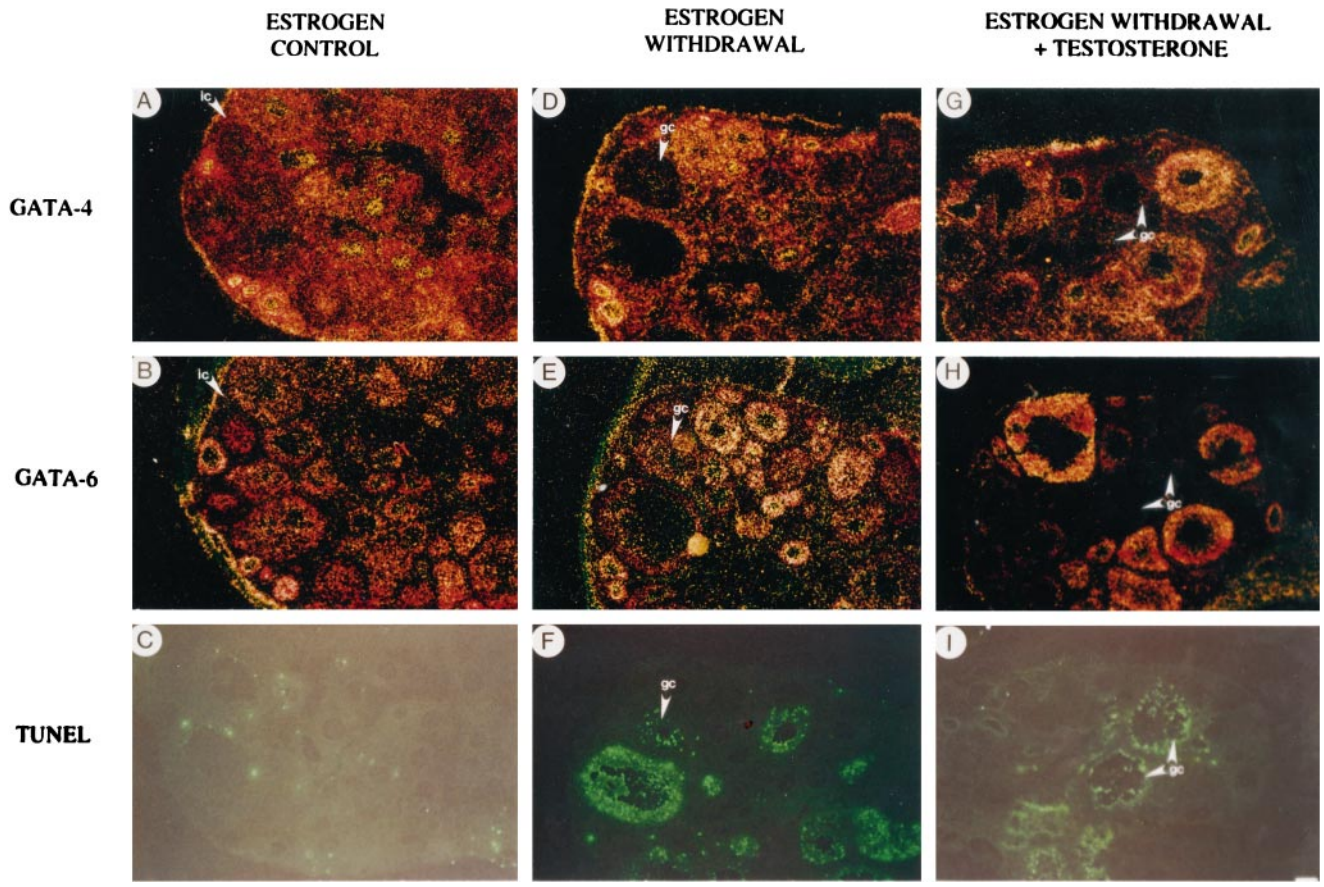

FIG. 9. GATA-4 and GATA-6 mRNA expression in granulosa cells undergoing apoptosis in response to estrogen withdrawal ± androgen administration. Immature (3-week-old) female mice were stimulated for 2 days with repeated injections of DES, followed by one of three treatments: (A–C) 2 additional days of DES (*i.e.* estrogen control), (D–F) no further treatment (*i.e.* estrogen withdrawal), or (G-I) 2 days of testosterone injections (*i.e.* estrogen withdrawal plus testosterone administration). Ovaries were then harvested and subjected to *in situ* assays for GATA-4 mRNA (A, D, G), GATA-6 mRNA (B, E, H), or apoptosis (C, F, I). gc, Granulosa cells (apoptotic); ic, interstitial cells.

the regulation of ovarian development. On the basis of these expression patterns, we propose that GATA-4 may control genes involved in maturation and/or maintenance of granulosa cells within early follicles (*i.e.* before ovulation). Alternatively, expression of GATA-4 may serve to prime early follicular cells for the transition to late maturation or apoptosis. The abrupt decrease in GATA-4 associated with ovulation or apoptosis indicates that this factor is not required for the late stages of follicular development, apoptosis, or luteinization. It is possible that GATA-4 functions as a cell survival factor in granulosa cells, analogous to the proposed role of GATA-1 as an anti-apoptosis factor in erythroid cells (23). Like GATA-4, transcription factor GATA-6 may regulate genes involved in granulosa cell function. That GATA-6 message is abundantly expressed in granulosa cells both preovulation and post ovulation suggests a unique function for this member of the GATA-binding family in the late stages of follicular development.

Previous studies have documented that GATA-4 and GATA-6 are coexpressed in a variety of tissues, including myocardium and gut epithelium (13, 19, 24–31). Overlap in the distributions of GATA-4 and GATA-6 transcripts in the heart, gut, and granulosa cells of the ovary raises the possibility of interplay between these two transcription factors. Members of the GATA-binding protein family have been shown to form homodimers (45, 46), heterodimers with other GATA-binding proteins (46, 47), and complexes with other classes of transcription factors (47, 48), including steroid hormone receptors (21, 22). Given their patterns of expression, it is conceivable that GATA-4 and GATA-6 associate with one another or with steroid hormone receptors in granulosa cells of primary and antral follicles, although at present there is no direct evidence to support this hypothesis. That the activity of the prototypical GATA-binding protein, GATA-1, can be modified by heterodimerization with the estrogen receptor (21, 22) raises that possibility that the activity of GATA-4 or GATA-6 in ovarian cells is regulated through interactions with steroid hormone receptors.

Of interest, the "erythroid" transcription factor GATA-1 has been shown to be expressed in a developmental- and stage-specific manner in Sertoli cells of the mouse testes (32, 33), although the target genes for GATA-1 in the testes have not been elucidated. Sertoli cells in the male are functionally analogous to granulosa cells in the female, suggesting that the role of GATA-1 in Sertoli cells may be similar to the role of GATA-4 or GATA-6 in granulosa cells.

Target genes for GATA-4 and GATA-6 in the ovary have not yet been established, but several genes expressed selectively in ovarian granulosa cells contain GATA motifs in

their promoters (10, 49), as discussed elsewhere (33). The genes encoding inhibinα and aromatase are of particular interest because these genes are involved in gonadogenesis and reproduction, are expressed in granulosa cells, and contain pairs of GATA sites that have been conserved across species. Whether GATA-4 and GATA-6 act as positive or negative regulators at these sites is currently unknown. Proof that these and other genes are bona fide targets for GATA-4 or GATA-6 *in vivo* awaits formal genetic tests [*e.g.* knockout studies or antisense inhibition experiments (50)].

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