Steroidogenic factor-1 (SF-1) (NR5A1) is an orphan nuclear receptor that plays a premier role in ovarian organogenesis. Recent studies document mRNA expression of the structurally related factor NR5A2 (FTF, LRH-1, SF-2) in the adult ovary and more specifically in granulosa cells and luteal cells but not theca cells. Conversely, SF-1 was shown to be expressed at higher levels in theca/interstitial cells. These latter observations raised the possibility that FTF/LRH-1 may control target gene expression in granulosa cells of developing follicles. Using quantitative PCR our results show that FTF/LRH-1 message is expressed at higher levels in the ovary than in liver or other tissues analyzed. We show by in situ hybridization and LacZ expression in ovaries of transgenic mice bearing an FTF-promoter-LacZ fusion gene that FTF/LRH-1 is selectively expressed in granulosa cells of rat and mouse ovaries and is not present in theca cells or interstitial cells. However, by a variety of approaches, we showed that SF-1 mRNA and protein are expressed in greater amounts than FTF/LRH-1 in granulosa cells of follicles at all stages of development. Expression of SF-1 mRNA and protein in granulosa cells was verified by in situ hybridization, immunohistochemistry of ovarian sections, and immunocytochemistry of cultured rat granulosa cells. The significance of SF-1 in regulating target gene activation was supported by EMSA. An abundant granulosa cell protein binding to the SF-1-binding motif (CCAAGGTCA) present in the aromatase promoter and an FTF/LRH-1 motif (TGTGCTTGAACA) in the α-fetoprotein promoter was supershifted by two SF-1-specific antibodies but not by an FTF antibody. Conversely, with the same probes, a less abundant protein/DNA complex present in liver and ovarian cell extracts was shifted by an FTF antibody but not by the SF-1 antibodies. SF-1 and FTF/LRH-1 were differentially regulated in vivo by estradiol, FSH and prolactin. Collectively these data indicate that granulosa cells of small and preovulatory follicles express both SF-1 and FTF/LRH-1 and that each orphan receptor may regulate target gene expression in these cells.

**Objective**: To investigate the differential expression of steroidogenic factor-1 (SF-1) and FTF/LRH-1 in the rodent ovary.

**Methods**: By using quantitative PCR, northern analysis, in situ hybridization, immunohistochemistry, and immunocytochemistry, the expression of SF-1 mRNA and protein in granulosa cells was assessed.

**Results**: SF-1 message was shown to be expressed in the ovary at higher levels than in liver. Northern analyses showed that FTF/LRH-1 transcripts were higher in the murine ovary than in the liver. Additionally, ovarian expression of FTF/LRH-1 was reported to be down-regulated in TAFI105 null mice that are infertile and display a severe ovarian phenotype. Furthermore, FTF/LRH-1 was cloned in equine ovarian tissue and shown to be expressed in granulosa cells but not theca cells of equine follicles. In contrast, SF-1 message was highest in the theca interstitial layer of the ovary.

**Conclusion**: SF-1 and FTF/LRH-1 are differentially regulated in the ovary, and SF-1 appears to be a key factor in regulating target gene expression in granulosa cells.

Abbreviations: AROM, Aromatase; Ct, cycle threshold value; E, 17β-estradiol; Fo, forskolin; FTZ-F1, fushi tazaru F1; GC, granulosa cell; GFP, green fluorescent protein; H, hyphophyes-cited; hCG, human chorionic gonadotropin; HE, preantral follicles; HEF, preovulatory follicles; PKA, protein kinase A; PMA, phorbol-12-myristate-13-acetate; PMSG, pregnant mare serum gonadotropin; qPCR, quantitative PCR; SF-1, steroidogenic factor-1; T, testosterone; WCE, whole-cell extract.
ulator of ovarian granulosa cell function during follicular growth in the adult.

A transgenic mouse-expressing green fluorescent protein (GFP) that is under the control of approximately 50 kb of SF-1 5′ flanking gene regulatory region exhibits strong GFP expression in theca and interstitial cells but not granulosa cells of adult mice (14). The localization of GFP was shown to be similar to that of endogenous SF-1 in the adrenal gland and testis as observed by immunohistochemistry. However, in these mice, ovarian expression, especially that in the embryonic ovary, does not mirror the pattern of expression of SF-1, suggesting that regulation of SF-1 is complex in the ovary. Hatano et al. (15) have previously shown using the same antibody that SF-1 is present at high levels in theca/interstitial cells of the adult rat ovary but that SF-1 is present in higher levels. However, this expression was hormonally regulated during follicular development (16). LRH-1 message and protein in the rodent ovary and whether SF-1 is expressed in significant amounts in granulosa cells as well as in theca/interstitial cells were not determined. Collectively, these data have in common that SF-1 is present in substantial amounts in the human ovary but that SF-1 is present in higher levels. However, cell-specific expression of SF-1 vs. FTF/LRH-1 in the human ovary was not determined. Collectively, these data have indicated that SF-1 was consistently high in theca/interstitial cells but not in granulosa cells. Conversely, the data indicated that FTF/LRH-1 might be more abundant than SF-1 in granulosa cells. If FTF/LRH-1, not SF-1, was a key regulator of granulosa cell function in the neonatal, prepubertal or adult ovary, differential roles for these two orphans in follicular growth could be emerging.

In the studies described herein, we sought to determine the relative and cell-specific expression of SF-1 and/or FTF/LRH-1 message and protein in the rodent ovary and whether this expression was hormonally regulated during follicular development and luteinization. For this we used a hypophysectomized (H) rat model as well as immature and adult cycling rats and mice. In situ hybridization analyses, immunohistochemical and immunocytochemical analyses, LacZ-knock-in expression, and Western blots and EMSAs were used to document cell- and hormone-specific expression patterns of FTF/LRH-1, compared with SF-1. Additionally, we sought to determine the relative amounts of SF-1 and FTF/LRH-1 transcripts in specific ovarian compartments using real-time quantitative PCR (qPCR). Our results showed that SF-1 is expressed in significant amounts in granulosa cells as well as in theca/interstitial cells of the rodent ovary. In contrast, although FTF/LRH-1 is expressed selectively in granulosa cells, the level of its message and protein (as determined by EMSA), compared with that of SF-1, was considerably less. Collectively, these results documented that SF-1, not FTF/LRH-1, is the predominant orphan receptor in granulosa cells of the immature and adult rodent ovary, whereas FTF/LRH-1 may play a more dominant role in luteal cells.

Materials and Methods

Animals

Immature female H rats (Harlan Sprague Dawley, Inc., Chicago, IL) were injected sc with 1.5 mg 17β-estradiol (E) or 0.2 ml propylene glycol (Sigma, St. Louis, MO) once daily for 3 d to stimulate the growth of large preantral follicles (HE). Over the next 2 d, rats were injected twice daily with 1.0 μg ovine FSH (NIH oFSH-16, National Hormone and Pituitary Agency, Rockville, MD)/0.1 ml PBS twice daily for 2 d to further stimulate growth of follicles (HEF). On the following morning, rats were injected ip with a single ovulatory dose (10 IU) of human chorionic gonadotropin (hCG) (Organon Special Chemicals, West Orange, NJ) to stimulate ovulation and luteinization (HEF/hCG). Following treatment with hCG, rats were injected twice daily (sc) with 100 μg ovine prolactin (National Pituitary Agency)/0.2 ml polyvinylpyrrolidone (Sigma). Ovaries were removed at specific time points. Ovaries from adult cycling and pregnant rats were also obtained. All ovaries were fixed in 4% paraformaldehyde (EM Science, Darmstadt, Germany) or granulosa cells were extracted as previously described (14). follicles were extracted and theca/interstitial cells soaked in 1.0 ml 0.1 M guanidinium thiocyanate, pH 7.4. Total RNA was isolated using TRIzol (Life Technologies, Inc., Grand Island, NY) and following the manufacturer’s protocol. Two hundred nanograms total RNA was reverse transcribed with random hexamer primer (PE Applied Biosystems, Foster City, CA) and avian myeloma virus-reverse transcriptase (Promega Corp., Madison, WI) for 1.5 h at 42 C in 80 μl reaction volume. One microcycler reverse transcription product was analyzed for SF-1, FTF/LRH-1, and 18S RNA expression by real-time PCR using the TaqMan universal PCR chemistry (Applied Biosystems) on an ABI Prism PE7700 sequence analyzer (Applied Biosystems). Gene-specific primers and probes were designed using Primer Express software (Applied Biosystems) and following manufacturer guidelines.

The ampiclon for SF-1 was 71 bp, extended over exons 3 and 4. Of rat/mouse SF-1 and consisted of the primers 5′-TCCCCTCTGCGCCCTT-C (Tm = 58 C) and 5′-CATTGATCGAGCCAGGA (Tm = 58 C) and the 5′-VIC and 3′-TAMRA-conjugated probe 5′-TGGGATCCTGCGCTT- GGAAGCT [Tm = 68 C, 45% granulosa cells (GC)]. Similarly, the rat FTF/LRH-1 ampiclon amplified 73 bp of rat FTF/LRH-1 extending over exons 2 and 3 by using the primers 5′-AACATGAGAAACACCCGG (Tm = 59 C) and 5′-GTCCCGGGCGGGAATG (Tm = 59 C) and the probe 5′-5′-FAM-AAAAGCTGCTGCGCTCCCGGACC-3′-TAR (Tm = 69 C, 64% GC). To verify the SF-1/LRH-1 data, another FTF/LRH-1 ampiclon amplified 76 bp of rat FTF/LRH-1 extending over exons 2 and 3 by using the primers 5′-AAATGTACGATGGAATGAA (Tm = 58 C) and 5′-GCCCGAATTTCTCTCCGC (Tm = 59 C) and the probe 5′-5′-FAM-TGGGCGCTACCTGCAAGGCCT (Tm = 68 C). A total of 600 nm primers and 200 nm probe were used in 50 μl reaction volume in MicroAmp 96-well plates. Thermal cycling conditions included 2 min AmpErase UNG activity at 50 C, 10 min AmpliTaq Gold DNA polymerase activation at 95 C, and 40 cycles of 15 sec at 95 C and 1 min at 60 C. Cycle threshold values (Ct) were analyzed using the SDS 1.9 software (Applied Biosystems). Singleplex SF-1 and FTF/LRH-1 quantities were normalized against 18S RNA amplification (primer/probe set, PE Applied Biosystems), for which input cDNA was diluted 50-fold. Relative expression levels were determined by the Ct method (ABI Prism 7700 SDS user bulletin, PE Applied Biosystems). The slope of log input amount vs. ΔCt was −0.00175 for SF-1 and −0.0152 for FTF/LRH-1, indicating similar amplification efficiency of SF-1, FTF/LRH-1, and 18S RNA reference.

In situ hybridization

Primers were designed to amplify rat SF-1: rat SF-1 F: CGAAGGTCATGTTATTTAAGG, rat SF-1 R: GTGTTGGCTAGTACACACGGC, rat/mouse FTF/LRH-1 F: TTGAACACAGGACCCTGCTTG, and rat/mouse FTF/LRH-1 R: GGAGCTTGAATGCGGTGTG, generating amplicons extending over exons 2 and 3 by using the primers 5′-AAATGTACGATGGAATGAA (Tm = 58 C) and 5′-GCCCGAATTTCTCTCCGC (Tm = 59 C) and the probe 5′-5′-FAM-TGGGCGCTACCTGCAAGGCCT (Tm = 68 C). The cDNAs were subcloned into the pCR-TOPO vector (Invitrogen, Carls-
electrophoretically transferred to 0.45-mm Immobilon membranes (Millipore Corp., Bedford, MA) and blocked for at least 1 h in Tris-buffered saline containing 5% milk and 0.1% Tween 20 and then incubated with a 1:1000 dilution of ABR SF-1 antibody (PA1–800). After three washes (10 min each), blot was incubated with 1:10,000 antirabbit horseradish peroxidase (Amersham Life Sciences, Piscataway, NJ) for 1 h at room temperature. Blots were washed again three times (10 min each), and the immunopositive bands were detected using the enhanced chemiluminescence assay system, (Pierce Chemical Co.) (20).

Transfection
Granulosa cells were harvested from intact rats primed with E (1.5 mg/0.2 ml progesterone glycol for 3 d), plated on serum-coated dishes in defined DMEM-F12 medium. Within 2–3 h the cells were transiently transfected with 1 μg of a reporter construct containing −3.9 kb/−79 bp of the FTF/LRH-1 promoter linked to luciferase (21, 25, 26). After 12–16 h, the cells were washed and cultured in fresh serum-free medium with or without FSH (50 ng/ml) and testosterone (T) (10 ng/ml), forskolin (F) (10 μM), phorbol-12-myristate-13-acetate (PMA) (20 nM) or F and PMA. Some cells were also treated with specific kinase inhibitors to determine the intracellular signaling cascades that impact FTF/LRH-1 promoter transactivation. The phosphatidylinositol 3-kinase inhibitor LY294002 (27–103–M005) was from Alexis (San Diego, CA). The protein kinase A (PKA) inhibitor H89 (371963) and the p38MAPK inhibitor SB203580 (593935) were from Calbiochem (San Diego, CA). After 4 h of agonist stimulation, the cells were lysed (0.2 M Tris (pH 8.0) containing 0.1% Triton X-100) and protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Inc.). Luciferase activity was analyzed as described previously (25). Data are expressed based on the amount of protein in each sample relative to light specific units per microgram protein (mean ± sd).

EMSAs
WCE protein was incubated for 30 min on ice with 50,000 cpm of end-labeled double-stranded oligonucleotide DNA probe with or without the competitor DNA in bandshift buffer [25% glycerol, 50 mM Tris (pH 7.5), 5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride + 10 μM aprotinin, 0.15 mg/ml BSA], 0.1 mM dithiothreitol, DDC, and NaCl] (24). Binding reactions were resolved by PAGE (0.25% × Tris-borate EDTA) and exposed to autoradiographic film. For the FTF/LRH-1-binding site (−175/−151 bp) in the aromatase (AROM) promoter an oligonucleotide 5'-GGAAGTTGTCTCCAAGATCCGTTTCC-3' and its reverse complement were used. For the SF-1 motif (−90/−64 bp) within the SF-1 promoter DNA-binding segment. Studies were carried out with heterozygous FTF/LRH-1+/− mice (FTF/LRH-1−/− null mice are embryonic lethal at E7.5). LacZ activity driven from the recombinant FTF/LRH-1 allele was detected essentially as detailed by Larochelle et al. (22). Ovaries were incubated overnight at 4°C in 30% sucrose in PBS and embedded in OCT before freezing. The 8-μm sections were stained at 30°C in PBS containing 1 mg/ml X-gal, 4 mM K4Fe(CN)6, 175 mM K3Fe(CN)6, 4 mM MgCl2, dehydrated without counterstaining, and mounted.

Statistics
A t test was used to analyze the mean ± sem for the qPCR and transfection data. Significant differences between treatment groups and samples (P < 0.01) has been denoted in the figures by a star.
adrenal, and ovary of immature intact rats. As shown in Fig. 1A, FTF/LRH-1 mRNA was higher in the ovary ($P < 0.01$), compared with expression in the pituitary gland and liver, the organ in which FTF/LRH-1 has been most rigorously studied (9). FTF/LRH-1 transcripts were detected at extremely low levels in the adrenal gland (Fig. 1A). In these same RNA samples, SF-1 message was higher in the adrenal gland ($P < 0.01$) than in the pituitary gland and the ovary. No SF-1 transcripts were detected in the liver (Fig. 1B). Likewise, when WCEs of ovarian, adrenal, and liver tissues were analyzed by Western blot, SF-1 was high in the ovary and adrenal samples but was absent in the liver (Fig. 1C).

To study the hormonal regulation and relative amounts of FTF/LRH-1 and SF-1 expression in the ovary, we used RNA samples from granulosa cells of female H rats treated with a sequential hormone regimen. The most striking feature of the data from this experiment is that SF-1 transcripts were expressed (based on the $\Delta$Ct values) at higher levels in all ovarian samples than were FTF/LRH-1 transcripts (Fig. 1D).

In granulosa cells from H rats, SF-1 expression was low. Treatment with E (HE) caused a 5-fold up-regulation ($P < 0.01$) of SF-1 message in granulosa cells relative to that in control H rats. Levels of SF-1 expression in granulosa cells remained high following treatment with FSH (HEF, GC). The residual ovarian tissue (comprised primarily of theca and interstitial cells) from HEF rats expressed a comparable amount of SF-1 message relative to granulosa cells (HEF; Fig. 1D). In luteinized ovaries of HEF rats treated with hCG for 72 h, SF-1 message was reduced ($P < 0.01$) and not significantly up-regulated in the corpora lutea by treatment with prolactin (Fig. 1D).

Expression of FTF/LRH-1 message was also low in granulosa cells from H rats and remained low following treatment with E (HE). Treatment with FSH (HEF) caused a 4-fold increase ($P < 0.01$) of FTF/LRH-1 in granulosa cells, compared with granulosa cells from HE rats. Levels of FTF/LRH-1 message were lower in residual ovarian cells from HEF rats, compared with granulosa cells from the same animals. In response to treatment with hCG, which facilitates luteinization, FTF/LRH-1 message decreased dramatically, however, cotreatment with prolactin restored FTF/LRH-1 expression in corpora lutea ($P < 0.01$). These data show that FTF/LRH-1 is differentially regulated in granulosa cells and corpora lutea by FSH and prolactin, respectively.

To study the cell type distribution of FTF/LRH-1 and SF-1 in a more physiological system, intact immature female rats were treated twice daily for 2 d with a low dose of hCG to stimulate the development of preovulatory follicles. Granulosa cells from the isolated preovulatory follicles expressed 20-fold higher levels of FTF/LRH-1 message than theca cells isolated from the same follicles ($P < 0.01$). Levels of SF-1 transcript expression were 4-fold higher in preovulatory granulosa cells than in preovulatory theca cells ($P < 0.01$). In granulosa cells, SF-1 mRNA was higher than FTF/LRH-1 ($P < 0.01$; Fig. 1E). Thus, in all ovarian samples examined, SF-1 expression appeared higher than FTF/LRH-1. Note also that levels of FTF/LRH-1 message were higher in granulosa cells from preovulatory follicles of intact rats, compared with the granulosa cells obtained from large antral follicles of HEF rats, suggesting that other pituitary factors may regulate FTF/LRH-1 expression in the ovary. Similar results were obtained for the relative expression of FTF/LRH-1 (in response to hormone treatments and in relation to SF-1) when a different set of primers and probe were used in the qPCR assay (see Materials and Methods, data not shown). Northern blots also determined that SF-1 expression was greater than that of FTF/LRH-1 in granulosa cells of HEF rats (data not shown).

Semi-quantitative RT-PCR analyses also revealed that FTF/LRH-1 is expressed in the neonatal rat ovary as early as the day of birth (data not shown).

**SF-1 and FTF/LRH-1 transcripts are expressed in different cell types in the rat ovary**

To study further the cell type-specific expression of FTF/LRH-1 and SF-1 in the rodent ovary, in situ hybridization was performed with ovarian sections from intact cycling and immature rats. As shown in Fig. 2, A and B, FTF/LRH-1 transcripts were expressed selectively in granulosa cells and corpora lutea but not in theca/interstitial cells in cycling adult and immature rats, respectively. This pattern closely mimics that of the qPCR data shown in Fig. 1, D and E. In contrast, SF-1 message appeared most abundant in the theca/interstitial cells in cycling and immature rats by this method (Fig. 2, G and H). This pattern differs from that of qPCR in which levels of SF-1 message were higher (~4-fold) in granulosa cells than in theca cells (Fig. 1E). The most likely explanation for this is that the granulosa cells used for preparing RNA analyzed by qPCR comprise a highly homogenous population of cells, whereas the theca cell layer contains many different cell types including fibroblasts, endothelial cells, and stromal cells that do not express SF-1 message. Therefore, although individual theca interna cells may have more SF-1 message per cell and appear more intense by in situ analyses, the theca layer isolated for RNA contains other cells not expressing SF-1. Given that the qPCR results indicate that FTF/LRH-1 transcripts are less abundant than those of SF-1, these results also show that the FTF/LRH-1 probe used for the in situ hybridization analyses is more sensitive than the SF-1 probe.

The hormonal regulation of FTF/LRH-1 and SF-1 during follicular growth was also analyzed by in situ hybridization using ovary sections from H rats treated with a sequential regimen of hormones. In H rats FTF/LRH-1 mRNA was observed distinctly in granulosa cells of primary and pre-antral follicles (Fig. 2C). In response to treatment with E (HE; Fig. 2D) and FSH (HEF) (Fig. 2E), FTF/LRH-1 mRNA continued to be localized to granulosa cells growing follicles. No detectable FTF/LRH-1 signal was observed in the theca/interstitial compartments of these ovaries (Fig. 2, D and E). After treatment with hCG to stimulate ovulation and luteinization, the signal for FTF/LRH-1 was low in corpora lutea but was retained in granulosa cells of small, nonluteinized follicles (Fig. 2F). The low signal of FTF/LRH-1 in the corpora lutea of the hormonally primed H rats is related to the absence of pituitary prolactin. As shown in Fig. 1D, prolactin is a critical component that regulates FTF/LRH-1 expression in the corpus luteum.

When SF-1 was analyzed in sections from these same tis-
FIG. 1. FTF/LRH-1 and SF-1 exhibit different patterns of expression and regulation. TaqMan qPCR was performed using amplicons designed specifically against rat SF-1 and FTF/LRH-1 as described in Materials and Methods. Values were calculated using the ΔCt method. In all graphs SF-1 is a gray bar, whereas FTF/LRH-1 is a solid black bar. For all experiments, 200 ng total RNA was reversed transcribed and subjected to qPCR. A, Total RNA was isolated from tissue samples: liver, pituitary gland, adrenal gland, and the ovary of immature untreated intact rats was reverse transcribed and analyzed by qPCR for FTF/LRH-1 and 18s. Graph represents FTF/LRH-1 values normalized to 18s. B, The same RNA samples used in A were analyzed by qPCR for SF-1. Graph represents SF-1 values normalized to 18s. C, WCEs (5 μg) of ovary, liver, and adrenal samples were analyzed by Western blot using an SF-1 specific antibody (ABR, PAI 800) diluted 1:1000. D, Total RNA was isolated from granulosa cells (GC) of H rats without hormone treatment or treated with estradiol (HE) and FSH (HEF) or total RNA from whole ovaries of HEF rats treated with hCG for 72 h without (HEF/hCG 72 h) or with treatment with prolactin (HEF/hCG 72 h + PRL) was reverse transcribed and subjected to qPCR. Graphs represent SF-1 or FTF/LRH-1 values relative to the internal control 18s. E, Total RNA from granulosa cells and theca cells isolated from preovulatory (PO) follicles of intact rats was reverse transcribed and subjected to qPCR. Graph represents the relative amounts of SF-1 or FTF/LRH-1 relative to 18s internal control. Each sample was run in triplicate and each experiment was run at least twice with highly reproducible results. The values represent the mean ± SEM of two experiments. A t test was used to determine significant differences among hormone treatments or tissue types. The star denotes significance (P < 0.01), compared with the preceding group.
sues, the SF-1 signal was detected in the theca and interstitial cell compartments of H ovaries, but no obvious signal was detected in granulosa cells (Fig. 2I). However, following treatment with E (HE), SF-1 mRNA was clearly expressed in granulosa cells of most large preantral follicles (Fig. 2J). SF-1 mRNA was also detected in some granulosa cells as well as theca cells of large antral (preovulatory) follicles of HEF rats (Fig. 2K). After treatment with FSH and hCG (HEF, hCG), SF-1 message was detected in newly formed corpora lutea (Fig. 2L).

**SF-1 protein is localized to granulosa cells, interstitial cells, and corpora lutea**

To further characterize the cell type-specific expression and localization of SF-1 immunohistochemical analyses were performed using rat ovarian tissues. In ovaries from immature rats, SF-1 staining was present and appeared to be nuclear in granulosa cells of some (but not all) follicles and was clearly more abundant and nuclear in theca/interstitial cells (Fig. 3, A and B). Ovaries from H rats exhibited a unique staining pattern with SF-1 being localized in the theca/
FIG. 3. SF-1 protein is localized to nuclei of granulosa cells, theca/interstitial cells, and corpora lutea. A, Immunohistochemistry was performed with ovarian sections from intact immature rats and hormonally primed H rats using an SF-1 antibody (generously provided by Ken Morohashi) at a concentration of 1:750. Open arrows denote SF-1 expression in granulosa cells; closed arrows indicate SF-1-positive theca/interstitial cells. Similar results were observed with the ABR antibody (data not shown). B, At higher magnification (×40 and ×63 objective), nuclear localization of SF-1 in granulosa cells (open arrow) and theca cells (solid arrow) is evident in a healthy follicle, compared with an atretic follicle. It is important to note that in granulosa cells the nuclear to cytoplasmic ratio is high (i.e., most of the cell is nucleus). Clear (nonstained) areas surrounding the nuclei represent the granulosa cell cytoplasm. The dashed line represents the basal lamina (BL). C, WCEs (5 μg protein/ lane) prepared from ovaries of immature rats and granulosa cells of hormonally primed H rats were analyzed by Western blot using an SF-1 specific antibody from ABR (1:1000). These results confirm the presence of SF-1 protein and its regulation by E and FSH in granulosa cells of intact immature rats and the H rat model. D, Immunocytochemistry was performed using the SF-1 antibody from ABR at a concentration of 1:50 with rat granulosa cells cultured without (control, T = 0) or after treatment with FSH (50 ng/ml) and T (10 ng/ml) for 48 h. Fluorescence was detected using an Axioplan2 microscope. E, SF-1 protein (as detected by the ABR antibody) was expressed in granulosa cells of intact immature and PMSG-treated mouse ovaries as well as the decrease of SF-1 within 4 h following hCG and in whole ovaries (WO) of PMSG/hCG-treated mice that contain predominantly corpora lutea.
interstitial cells as well as in the oocytes but was not readily apparent in granulosa cells. The localization of SF-1 in oocytes likely represents nonspecific staining to oocytes that we have observed with other antibodies because no signal was observed in the in situ hybridization analyses. Following treatment with estradiol (HE) and FSH (HEF), immunoreactive SF-1 was observed in granulosa cells of growing follicles. In accordance with the in situ hybridization data, nuclear staining of SF-1 was present but low in corpora lutea from HEF rats treated with hCG for 24 h. Granulosa cells and theca/interstitial cells in the HEF hCG 24 h-treated rats remained immunopositive for SF-1. In images from an ovary from an immature intact rat taken at higher magnification, SF-1 is localized clearly in nuclei (compared with the pale staining of surrounding cytoplasm) of granulosa cells and theca cells (Fig. 3B, ×40 and ×63). Note also that SF-1 staining in granulosa cells of a healthy follicle is greater than that in an adjacent atretic follicle, indicating differential expression of SF-1 in follicles (Fig. 3B, ×40).

Using an SF-1 antibody (ABR), we show that a major immunoreactive band (~53 kDa) corresponding to the predicted size of SF-1 is present in WCE of ovaries from intact immature rats as well as in granulosa cells from H rats (Fig. 3C). Furthermore, immunoreactive SF-1 is increased in granulosa cells of H rats treated with E (HE) and FSH (HEF) and decreased in response to hCG confirming the qPCR data for SF-1 (Fig. 1). The other minor immunoreactive bands are likely to be nonspecific because the larger band does not correspond to the pattern of FTF/LRH-1 expression (Fig. 1) nor does the ABR antibody supershift FTF/LRH-1 in EMSAs (see Fig. 5).

To determine whether SF-1 was expressed and hormonally regulated in isolated granulosa cells, immunofluorescent analyses were performed with cultured rat granulosa cells. Using the ABR anti-SF-1 antibody, we show that SF-1 was expressed at low levels in the nuclei of granulosa cells cultured in medium alone overnight (T = 0) control (Fig. 3D) or for 48 h (data not shown). Following treatment with FSH and T for 48 h (FSH/T, 48 h), which causes granulosa cell differentiation, SF-1 was strongly evident in the nuclei of all granulosa cells, suggesting that SF-1 levels in nuclei increase in these differentiated cells (Fig. 3D). Similar results were observed with the antibody from Dr. K. Morohashi (data not shown). Thus, the SF-1 is localized to nuclei of granulosa cells before and after exposure to FSH in vitro.

To determine whether the immunolocalization of SF-1 in the mouse ovary was similar to that in the rat, immunohistochemical analyses were done (Fig. 3E). As shown, SF-1 localizes not only to granulosa cells but also to theca cells of growing follicles in the immature and adult mouse ovaries. SF-1 immunostaining was also present in lutea cells within the adult ovary. Western blot analyses of SF-1 in mouse ovarian extracts documented further that SF-1 is expressed and regulated by hormones. Thus, in the mouse and rat ovary, the patterns of expression of these two orphan receptors are highly similar.

**FTF/LRH-1 is localized specifically to granulosa cell**

Mice null for FTF/LRH-1 die during embryogenesis at E 7.5. Therefore, any study of the function of this orphan receptor in the neonatal and adult ovary is precluded. However, because the FTF/LRH-1 null mice were made with an FTF-LacZ knock-in approach, they afford an important mechanism to study the cell type expression of FTF/LRH-1 in ovaries of mice with one allele of the FTF/LRH-1 gene driving expression of LacZ. By staining sections of adult mouse ovaries with X-gal, the expression of LacZ was specifically and robustly localized in granulosa cells of follicles at many stages of development, i.e. from small preantral to large antral stages (Fig. 4A). LacZ staining was not observed in theca or interstitial cells indicating that the FTF/LRH-1 locus is silent in these tissues. In situ hybridization analyses confirmed exclusive localization of FTF/LRH-1 message in granulosa cells of small growing follicles and in preovulatory follicles of immature and pregnant mare serum gonadotropin (PMSG)-treated mice, respectively (Fig. 4B). FTF/LRH-1 message was also detected in granulosa cells and corpora lutea of PMSG, hCG-treated mouse ovaries as well as in
ovaries of pregnant mice (Fig. 4B). The strong signal for FTF/LRH-1 message in corpora lutea of pregnant rats is consistent with the regulated expression of this gene by prolactin as shown by qPCR analyses in Fig. 1.

To document that the FTF/LRH-1 promoter used in the Lac Z knock-in mice was active in granulosa cells and could be regulated by hormones and signaling cascades that are downstream of FSH, an FTF/LRH-1 luciferase
The construct was transfected into granulosa cells (Fig. 4C). Transfected cells were cultured in the presence or absence of FSH (50 ng/ml) and T (10 ng/ml); Fo (10 μM), a direct activator of adenyl cyclase; PMA (20 nM), a direct activator of protein kinase C or Fo; and PMA. In some wells, specific protein kinase inhibitors were added with the agonists, and the cells were harvested after 4 h. H89 was added to block PKA, SB203580 to block p38 MAPK, and LY294002 to block the PI3-kinase pathways (27). The FTF/LRH-1 transgene was highly induced by hormones and cAMP signaling cascades in granulosa cells (Fig. 4C). Luciferase activity increased in granulosa cells treated with FSH/T (15-fold; P < 0.01), Fo (8-fold; P < 0.01), or Fo and PMA (26-fold; P < 0.001), compared with nontreated controls. PMA alone had little or no effect on the activity of this transgene, indicating that the protein kinase C pathway alone could not directly activate the promoter. However, PMA enhanced the activation by agonists that stimulate cAMP (Fig. 4B). Furthermore, activation of the FTF/LHR-1-luciferase reporter by FSH/T was inhibited 60–85% by H89 and 40–50% by a PI3-kinase inhibitor LY294002. Induction by Fo or Fo and PMA was blocked approximately 50% by a p38 MAPK inhibitor SB203580. Thus, transactivation of this gene in granulosa cells is robust and affected by several signaling pathways with PKA and p38 MAPK exerting the most dramatic effects.

Relative amounts of FTF/LRH-1 and SF-1 in the ovary and liver

To determine the relative amounts of FTF/LRH-1 and SF-1 protein present in rat liver and ovarian tissues, EMSAs were run using WCEs from rat liver, immature ovaries, and preovulatory granulosa cells. Oligonucleotides corresponding to the regulatory regions of the AFP and AROM promoters known to bind FTF/LRH-1 and SF-1, respectively, were synthesized and used as the labeled probes (9, 28). In the first experiment, the binding pattern of liver extract protein was compared with that of ovarian extracts using both the AFP and AROM probes. When liver extracts were incubated with either the AFP or AROM probe, two protein/DNA complexes (designated II and III) were formed and showed similar migration profiles (Fig. 5A, lanes 1 and 8). Formation of the protein/DNA complexes was reduced/blocked by a 20-fold excess of cold competitor DNA (Fig. 5A, lanes 2 and 9). These results confirm previously published analyses of FTF binding to the AFB probe (9, 21). In addition, antibodies to SF-1 (K. Morohashi) did not alter any band in the liver extracts (Fig. 5A, lanes 3 and 10). In contrast, when ovarian extracts were reacted with the AFP or AROM probes, two similar complexes (II and III), as well as three additional complexes (I, IV, and V) were observed (Fig. 5A, lanes 4–7 and 11–14). Nonspecific IgG did not alter any of the ovarian

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**Fig. 5.** Proteins in liver, ovary, and granulosa cells bind to the SF-1 motif of the aromatase promoter and the FTF/LRH-1 motif of the AFP promoter. A, EMSAs were performed with radiolabeled AFP promoter (−175/−151) (lanes 1–7) and AROM promoter (−90/−64) (lanes 8–14) probes. Extracts from rat liver (20 μg) (lanes 1–3 and 8–10) and ovary (1.5 μg) (lanes 4–7 and 11–14) were incubated for 45 min on ice in the presence of 20-fold excess of cold competitor DNA (lanes 2 and 9), nonspecific IgG (lanes 5 and 12), or SF-1 antibodies from K. Morohashi (lanes 3, 6, 10, and 13) or ABR (lanes 7 and 14). A total of 2 μl IgG and SF-1 antibodies were used. Solid arrows designate the FTF/LRH-1 band (III), the bracket indicates the SF-1 bands (IV/V), and the open arrowheads denote the SF-1 supershifts (lanes 6, 7, 13, and 14). B, Liver (20 μg) (lanes 1–3), ovary (1.5 μg) (lanes 4–6), and preovulatory granulosa cells (2.4 μg) (lanes 7–8) were incubated on ice with labeled AROM probe without or with 20-fold excess of cold competitor DNA (lanes 2, 4, 8), FTF antibody (lanes 3 and 6). Solid arrows designate the FTF/LRH-1 band (III), the bracket indicates the SF-1 bands (IV/V), and the solid stars denote the FTF/LRH-1 supershifts (lanes 3 and 6). C, Ovarian extracts (2 μg) were incubated with the FTF/LRH-1 probe without (lanes 2) or with a 20-fold excess of unlabeled competitor AFP DNA (lanes 3). Additional extracts were reacted with either a nonspecific IgG (lane 4), an FTF antibody (lanes 5) or the ABR SF-1 antibody (lane 6). Solid arrows designate the FTF/LRH-1 band (III), the bracket indicates the SF-1 bands (IV/V), the solid stars denote the FTF/LRH-1 supershifts (lane 5), and the open arrowhead denotes the SF-1 supershift (lane 6). Lane 1 is probe alone.
complexes (lanes 5 and 12), whereas antibodies to SF-1 (K. Morohashi and ABR) selectively supershifted the major complexes IV and V but did not alter the formation of the upper complexes (I–III) (Fig. 5A, lanes 6, 7, 13, and 14). Complex I appeared to be nonspecific. These results show that the ovary sample contains SF-1, whereas the liver does not, confirming the qPCR and Western blot data presented in Fig. 1, B and C. In addition, the data show that SF-1 binds to both the AFP and AROM sites.

To determine whether the bands (II and III) observed in liver and ovary contained FTF/LRH-1, additional EMSAs were done. As shown in Fig. 5B, liver, ovary, and highly purified granulosa cell extracts were reacted with the AROM probe. Complexes II and III were formed by liver, ovary, and granulosa cell extracts (Fig. 5B, lanes 1, 5, and 7, respectively). These bands were competed with a 20-fold excess of competitor DNA (Fig. 5B, lanes 2, 4, and 8, respectively). Furthermore, when an antibody to FTF/LRH-1 was included in the reaction, the lower, more intense band present in liver extracts (Fig. 5B, lane 3) as well as complex III present in ovarian extracts (Fig. 5B, lane 6) were supershifted (denoted by the star). No supershift was observed when a control IgG was used (Fig. 5B, lane 5). To confirm the supershift of FTF/LRH-1 in the ovarian extracts, an additional experiment was done using the AFP probe. As shown in Fig. 5C, no complexes were formed with probe alone (lane 1), whereas the addition of ovarian extracts produced complexes I–V that were reduced with a 20-fold excess of AFP probe (lanes 2 and 3, respectively). A nonspecific IgG did not alter complex formation (lane 4).

However, when the FTF antibody was added to the reaction, band III was supershifted (Fig. 5C, lane 5), whereas when the ABR SF-1 antibody was included in the reaction, only complexes IV and V were supershifted (Fig. 5C, lane 6). Collectively, these results show that SF-1 remains the predominant binding species in the ovarian and granulosa cell extracts, whereas FTF/LRH-1 protein is present but at lower amounts in these same extracts. Conversely, FTF/LRH-1 but not SF-1 is present in liver cells.

Discussion

The discovery of SF-1 (NR5A1) and the documentation of its premier role in the organogenesis of the ovary, pituitary, and adrenal (4) set the stage for intensive investigations of its role in controlling gene expression in each of these tissues (5). However, recent studies document the expression of the closely related factor NR5A2 (FTF, LRH-1, SF-2) in the ovary (1) and more specifically in granulosa cells and luteal cells but not theca cells (2). These observations have challenged the dogma that SF-1 is the key orphan receptor controlling target gene expression in granulosa cells. Using qPCR and EMSAs, the results presented herein verify that FTF/LRH-1 mRNA and protein is expressed at equal or higher levels in the ovary than in liver or any of the tissues analyzed. We also confirm by in situ hybridization and LacZ knock-in expression that FTF/LRH-1 is selectively expressed in granulosa cells of rat and mouse ovaries and is not present in theca cells or interstitial cells. These data show that the absence of FTF/LRH-1 mRNA in theca/interstitial cells by in situ analyses is not a detection problem. Rather, the FTF/LRH-1 locus is silent in theca and interstitial cells. Despite our initial in situ hybridization data indicating that FTF/LRH-1 might be higher in rodent granulosa cells than SF-1 (29), we document for the first time that SF-1 mRNA and protein are expressed in greater amounts than FTF/LRH-1 in granulosa cells of rat follicles at all stages of development. These results indicate further that the probe used for the in situ hybridization analyses of FTF/LRH-1 are more sensitive than that for SF-1, thereby showing that in situ hybridization cannot always be a reliable method of comparing the relative amounts of endogenous message.

Specifically, we show by qPCR that SF-1 mRNA is considerably higher than FTF/LRH-1 mRNA in granulosa cells of small, growing follicles in immature and adult rats as well as in granulosa cells of hormonally primed hypophysectomized rats. Expression of SF-1 mRNA in granulosa cells was verified by in situ hybridization of rat ovaries. Expression of SF-1 protein in granulosa cells was verified by Western blots, immunohistochemistry of ovarian sections, and immunocytochemistry of cultured rat granulosa cells. The functional significance of SF-1 in regulating target gene activation was suggested by showing that the major granulosa cell protein binding to the consensus SF-1 motif present in the aromatase promoter was supershifted by SF-1-specific antibodies. These results are in agreement with those of Hatano et al. (15) and Takayama et al. (30), who showed by immunofluorescent studies that SF-1 was present in granulosa cells of immature rats. That SF-1 is not evenly expressed in all follicles of adult rats or mice most likely reflects the fact that many follicles are in different stages of development and/or atresia. The absence of detectable expression of an SF-1 promoter-GFP construct in the follicles of adult mice as reported recently by Parker’s group is puzzling but may reflect the more restricted expression in of SF-1 in granulosa cells of specific follicles (atretic vs. growing; see Fig. 3B) of adult mice, compared with immature mice (14).

Collectively, our results suggest that both SF-1 and FTF are potential regulators of granulosa cell gene expression in the mouse and rat. Our results further document that expression of these two genes in granulosa cells is regulated by distinctly different hormonal conditions. SF-1 mRNA and protein was increased in granulosa cells of H rats predominantly by E, whereas FTF/LRH-1 was increased slightly by E and more by FSH in granulosa cells and by prolactin in luteal cells. This differential regulation by hormones is not surprising because the promoters of these two genes are distinctly different. SF-1 has been shown to be regulated by WT-1, Sox-9, and Lhx-9 in the embryonic gonad and Sertoli cells (31, 32). Mice null for WT-1 or Lhx-9 fail to develop gonads (33, 34). In adult testis SF-1 is regulated at least in part by USF, NF-Y, and Sp1/Sp3 (35, 36). Because USF (37), Sp1/Sp3 (16), and NF-Y proteins (unpublished data) are highly expressed in granulosa cells and known to regulate expression of other genes, it is likely that in granulosa cells as in Sertoli cells these proteins participate in the regulation of SF-1 (38). FTF/LRH-1 null mice are embryonic lethal precluding any analysis of the role of this factor in gonadogenesis. However, because SF-1 null mice fail to develop gonads, one might...
predict that FTF/LRH-1 is either not expressed significantly in the embryonic gonad or does not have redundant functions of SF-1.

What regulates the transcriptional activation of FTF/LRH-1 in the ovarian granulosa cells is not yet entirely clear. The FTF/LRH-1 promoter has been shown to be regulated in liver cells by GATA- and FTF/SF-1-binding motifs (9, 21). Because SF-1 and GATA-4 are present in granulosa cells (1, 2, 5) and are phosphorylated in response to specific agonists (39, 40), these factors may transactivate the FTF/LRH-1 promoter activity in granulosa cells via their respective regulatory motifs. By transfection analyses we show that PKA and p38 MAP kinase more than PI3-kinase signaling pathways impact transactivation of the FTF/LRH-1 reporter gene in granulosa cells. Furthermore, the increase in FTF/LRH-1 mRNA in granulosa cells parallels the increase in GATA-4 protein (data not shown), suggesting that GATA-4 might act synergistically with FTF/LRH-1 or SF-1 in granulosa cells of small growing follicles to up-regulate FTF/LRH-1. That prolactin up-regulated FTF/LRH-1 in luteinized cells and the FTF/LRH-1 signal in corpora lutea of pregnant mouse ovaries was intense indicate that the Janus kinase/ signal transducer and activator of transcription pathway may be involved (24).

Are there specific functional roles for SF-1 and FTF/LRH-1 in the ovary? Clearly our results as well as those of others (15, 41) document that SF-1 but not FTF/LRH-1 is expressed in theca cells and interstitial cells and therefore is the factor impacting genes dependent on the orphan receptor regulatory motif in these tissues. The relative and specific roles of SF-1 and FTF/LRH-1 in granulosa cells are far more difficult to predict. The DNA binding domains of FTF/LRH-1 and SF-1 are highly homologous (~88%) and SF-1 binds equally well to the SF-1 consensus found in the aromatase promoter (CCTAAGGTCA) and the FTF/LRH-1 motif of the AFP promoter (TGTTCAAGGACA) (Fig. 5). In this regard both SF-1 and FTF/LRH-1 have been shown to activate genes similarly; namely the hepatitis B viral promoter (42) and aromatase promoter (12) as well as several other steroidogenic enzymes (13) in transfection experiments. Furthermore, the phosphorylation of SF-1 serine 203 facilitates recruitment of cofactors as well as the stabilization of the ligand binding domain (39, 43). This serine is conserved in FTF/LRH-1 (9, 39, 43). Thus, DNA binding and activation of these two orphan receptors looks similar, at least at the biochemical level. Whether or not SF-1 and FTF/LRH-1 are equally effective in vivo is not yet known and will depend not only on the levels of each orphan receptor but also on their specific interactions with known corepressors such as DAX-1 and SHP (10) and specific coactivators. If, for example, a majority of SF-1 is repressed normally by DAX-1 whereas FTF/LRH-1 is not, the relative amount of functional FTF/LRH-1 may equal or exceed that of SF-1 in the intact granulosa cells. To address fully the roles of SF-1 and FTF/LRH-1 in the ovary, it will be necessary to generate ovarian specific knockouts of each orphan as well as both together. These models will help define specific downstream targets and functions of these transcription factors in the ovary.


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