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M.D. Li
USDA-ARS

L.V. DePaolo
Whittier Institute for Diabetes and Endocrinology

J.J. Ford
USDA-ARS

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M.D. Li,[3,5] L.V. DePaolo,[4,6] and J.J. Ford[2,5]

USDA-ARS,[5] RLH U.S. Meat Animal Research Center, Clay Center, Nebraska 68933
The Whittier Institute for Diabetes and Endocrinology,[6] La Jolla, California 92037

ABSTRACT

Expression of the follistatin (FS) and inhibin/activin (I/A) α, β_A, and β_B subunit genes in porcine ovarian follicles was evaluated by reverse transcriptase polymerase chain reaction and/or RNase protection procedures to establish changes during the final stages of follicular development. For the I/A α and β_A subunits, expression increased ($p < 0.05$) as follicles progressed to the mid-stage of the follicular phase. The β_B subunit was expressed in lower concentrations, and all three I/A subunits showed a marked reduction ($p < 0.01$) in expression by the late stage of follicular development. In contrast to this pattern, FS gene expression decreased ($p < 0.05$) as follicles developed from the early (low estradiol) to the mid stage (high estradiol) and continued to decline in advanced follicles (after estrus). The predominant mRNA encoded for FS-315, and the ratio of mRNA for FS-315 to mRNA for FS-288 did not differ significantly during the three stages. Within an animal, concentration of FS mRNAs was related more to stage of the follicular phase than to follicular size. Follicular fluid concentration of FS changed in a manner similar to that observed for expression of its gene. We conclude that expression of the FS gene and translation of its mRNA decrease as follicles approach ovulatory status.

INTRODUCTION

Follistatin (FS) is a monomeric protein originally isolated from bovine and porcine follicular fluids and was shown to be an inhibitor of FSH secretion [1–3]. Molecular cloning and sequence analysis of FS cDNAs and genes from pigs [4, 5], humans [6], and rats [7] revealed that the FS gene is highly conserved in these species, and there are two forms of FS mRNA generated by alternative splicing [4]. The predicted protein sequences of the secreted forms consist of 288 (FS-288) and 315 (FS-315) amino acids. The FS-315 differs from FS-288 by having an extra 27 amino acids at the carboxyl terminus. The FS gene is a single copy of approximately 6.0 kilobases (kb) consisting of five exons and an initial exon encoding the signal peptide [4].

One biological function of FS, inhibition of FSH secretion, is similar to that of inhibin; but FS is structurally quite different, and its potency is weaker than that of inhibin. FS binds both activin [8] and inhibin [9] through a common β subunit. This binding inhibits activin's stimulation of FSH secretion [9]. Although FS mRNA has been found in a variety of tissues, including ovary, testis, and pituitary [4, 10–12], regulatory effects within these tissues are poorly understood. Northern blot hybridization of porcine ovarian poly(A)$^+$ RNA showed a single band of 2.5 kb, but work from other laboratories [11, 13, 14] using total RNA from rat and bovine tissues, revealed two bands of approximately 2.5 kb and 1.6 kb, respectively. Two major (ca. 2.7 kb and 1.5 kb) and one minor (0.5 kb) mRNA for FS were detected in sheep [15].

Activins and inhibins were initially isolated from porcine and bovine follicular fluids as gonadal proteins with the capacity to stimulate and inhibit, respectively, FSH secretion and its synthesis in anterior pituitary glands. Inhibins are dimeric proteins consisting of a common α subunit and one of two β subunits, and activins are also dimeric proteins but are composed of only the β subunits [16, 17]. The transcripts for these subunits have been detected in gonads and several extragonadal tissues. In porcine follicles, only mRNA for the α and β_A subunits were detected by dot-blot analysis [18].

How FS gene expression is regulated during porcine follicular development remains to be characterized. A better understanding of such regulation will provide insight into the control of follicular selection and growth. The major objectives of the study reported here were 1) to determine how the expression of the FS gene differs in follicles of different sizes within the same animal, and of the same size but at different stages of the follicular phase of the estrous cycle, and 2) to determine the ratio of mRNA species coding for FS-315 to mRNA species coding for FS-288 as influenced by follicular size or stage of the follicular phase.

MATERIALS AND METHODS

Collection of Ovarian Follicles and Follicular Fluid

Females of mixed breeds (7–9 mo old) were evaluated for estrous behavior twice daily with a sexually mature male. All females had experienced at least one estrous cycle before they were slaughtered at random from 17 to 20 days after estrus (early to mid stages of the follicular phase, n = 12) or > 24 h after estrus (late stage of the follicular phase, n = 5). A jugular blood sample was obtained 24 h before and at slaughter for determination of plasma estradiol and progesterone concentrations [19, 20]. At slaughter, females were electrically stunned, and ovaries were obtained quickly and placed into cold saline. Follicles larger than 3 mm in diameter were dissected, with diameter determined with calipers. Each follicle was stored individually in liquid nitrogen except four to six that were representative of mean diameter for each animal. From these, follicular fluid was collected by aspiration and evaluated within a single assay for estradiol concentration [19].

Follicular fluid samples from an independent population of primiparous, crossbred females (> 1 yr old) were evaluated within a single assay for FS concentration by RIA [21]. Ovaries were obtained at slaughter, and follicular fluid was obtained by aspiration of the 16–20 largest antral fol-

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[1]Mention of names is necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the same by USDA implies no approval of the product to the exclusion of others that may also be suitable.

[2]Correspondence: J. Ford, USDA-ARS, RLH U.S Meat Animal Research Center, P.O. Box 166, Clay Center, NE 68933. FAX: (402) 762–4148; e-mail: ford@marcvm.marc.usda.gov

[3]Current address: Minneapolis Med. Res. Foundation, Dept. of Med., Hennepin County Med. Ctr., University of Minnesota, Minneapolis, MN 55404.

[4]Current address: NICHD, Bethesda, MD 20892.

TABLE 1. Ovarian status of females allotted to each group by cluster analysis.

Group	N	Follicle number	Follicle diameter (mm)	Follicular fluid E_2 (pg/ml)	ΔE_2[a] (pg/ml)	ΔP_4[a] ng/ml
I	4	20.8 ± 2.2	4.6 ± 0.2	14 ± 9	1.3 ± 0.6	−3.8 ± 1.6
II	8	20.1 ± 1.8	6.5 ± 0.2	334 ± 66	11.6 ± 1.6	−0.1 ± 0.2
III	5	15.0 ± 1.6	7.4 ± 0.3	52 ± 28	−8.8 ± 1.6	0.4 ± 0.1

[a] Changes in plasma E_2 and P_4 concentrations during the 24 h before collection of follicles.

licles per female. Porcine FS, a mixture of three molecular-weight forms, was the reference preparation. Estradiol concentrations were determined for follicular fluid, and sows were grouped according to criteria established in the cluster analysis (see *Statistical Analysis* section). The 27 females in the early phase of follicular development lacked active corpora lutea, had a mean follicular diameter of < 5.5 mm, and had follicular fluid estradiol concentrations of < 100 ng/ml. The 18 females in the mid-follicular phase had a mean follicular diameter of ≥ 5.5 mm, had follicular fluid concentrations of estradiol of > 100 ng/ml, and had not exhibited behavioral estrus. Sixteen females that were designated as being in the late phase of follicular development had exhibited behavioral estrus and had follicular fluid estradiol concentrations of < 150 ng/ml.

Primers Used in Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and Subcloning

On the basis of the reported nucleotide sequence [5], primers 5′-FS, 3′-FS1, and 3′-FS2 corresponding to nucleotides 1000–1029 (exon 3), 1837–1866 (exon 4), and 1939–1968 (exon 5) of the porcine FS gene were synthesized by the DNA Synthesis Laboratory of University of Nebraska. The primer sequences for the FS and β-actin genes were the same as those described previously [22]. The expected RT-PCR product amplified with 5′-FS and 3′-FS1 is 216 base pairs (bp) from both RNA species, and the product amplified with 5′-FS and 3′-FS2 is 318 bp from FS-315. Using these specific primers, amplified PCR products from cDNA that had been synthesized by reverse transciption were subcloned, sequenced, and determined to correspond to the predicted region of each gene [22, 23].

End-Labeling of Oligonucleotide Primers and Preparation of Riboprobe

Oligonucleotide DNA primers were labeled as described [24, 25]. After linearization of plasmids pFS-9, pα-9, $p\beta_A$-21, $p\beta_B$-5, and pβ-actin with *Eco*RV or *Hin*dIII, sense and antisense riboprobes were transcribed with DNA-dependent SP6 or T7 RNA polymerase in the presence of [α-^{32}P]uridine triphosphate (UTP) under the conditions described [24, 25]. The DNA templates were removed by incubation with RNase-free DNase I at 37°C for 15 min.

RNA Isolation and Quantitative RT-PCR

The 66 representative follicles with a variety of diameters were randomly chosen from the frozen follicles with at least one from each of the 17 gilts and consisted of 13 from group I, 42 from group II, and 11 from group III. Total cellular RNA was isolated from individual follicles by guanidine isothiocyanate extraction and CsCl centrifugation as described by Chirgwin et al. [26] and was treated with RNase-free DNase I at 37°C for 30 min before RT-PCR or RNase protection assay. Reverse transcription and quantitative PCR were performed [22, 24] for every gene studied. The amount of mRNA of each sample was normalized for porcine β-actin RNA, which was done simultaneously under the same conditions except with different primers.

RNase Protection Assay

Hybridizations and RNase digestions were performed with an RPA-II kit (Ambion, Inc., Houston, TX) [24, 25]. The numbers of follicular RNA samples included in the RNase protection assays were 5, 10, and 9 for early, mid, and late stages of the follicular phase with mean follicular diameters of 4.4 ± 0.2, 6.2 ± 0.3, and 9.3 ± 0.7 mm, respectively. Size of selected follicles represented the predominant size for each female. For every riboprobe used, two yeast RNA control tubes, one containing RNase A/T1 (negative control) and the other containing no RNase A/T1 (positive control), were always included in the assay.

Statistical Analysis

Data were analyzed with procedures CLUSTER and ANOVA (analysis of variance) in the Statistical Analysis System (SAS) statistical package [27–29] and are presented as mean ± SEM. Correlation coefficient (r_{ij}) was used to measure the similarities of different females because it could eliminate the effects on clustering of unit employed in each hormone measurement or follicle statistic. With the RT-PCR data, female nested within group (stage of the follicular phase) was used as error to test effect of group. To evaluate effect of size classification across groups, the data set was restricted to follicles ≤ 5 mm or > 7 mm. For evaluation of size classification within group, the data set was restricted to groups II or III, and follicle classification within gilt was the error term. When an *F*-test was significant, specific means were compared by using the Bonferroni procedure [29].

RESULTS

Classification of Animals

On the basis of plasma estradiol and progesterone concentrations on the day before and the day of follicle collection, plus mean and standard error of follicle number (> 3 mm in diameter) for each animal, three clustering techniques—average linkage, complete linkage, and centroid-hierarchical methods—were employed to group animals used in the study. Similar groupings resulted with each of these methods and classified the gilts into three groups, i.e., early (group I), mid (group II), and late (group III) stages of the follicular phase. Follicular statistics and steroid concentrations of the groups are summarized in Table 1. In group I, follicles were smaller (ca. 4.6 mm in diameter) and had lower estradiol concentrations than follicles observed in the other two groups. In group II, follicular size was

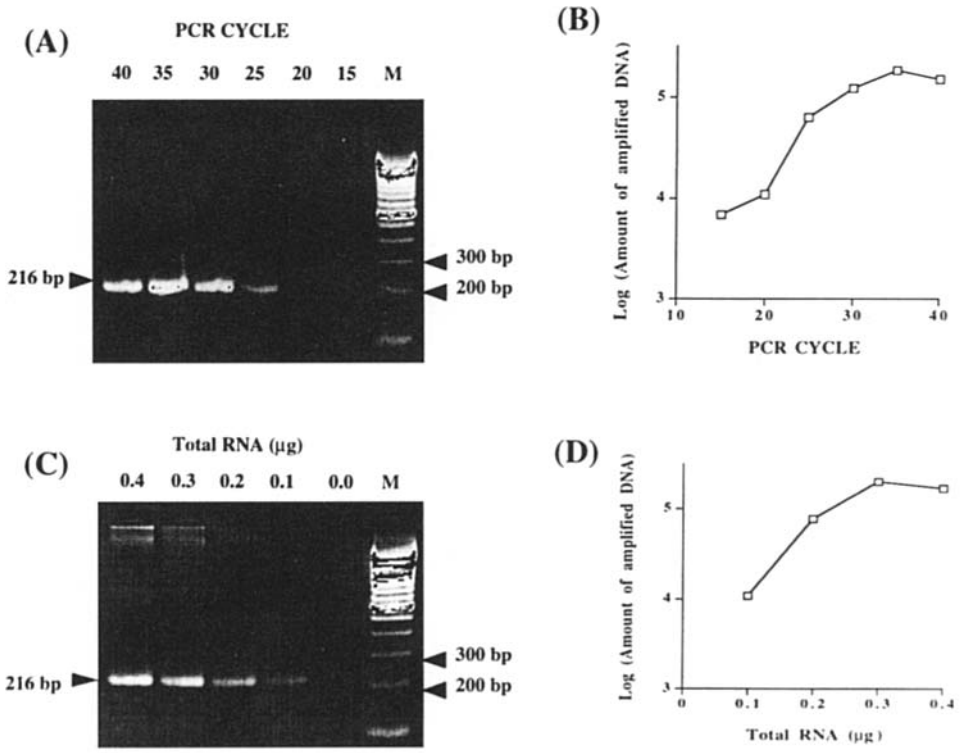

FIG 1 Increase in RT-PCR product with number of PCR cycles. Under the conditions given in the *Materials and Methods*, RNA from individual follicles was reverse transcribed, and cDNA mixture equivalent to 0.2 μg total RNA was subjected to various cycles of amplification (**A**), or aliquots of the cDNA mixture equivalent to 0.0–0.4 μg RNA were amplified for 25 cycles (**C**) with the primers 5′-FS and 3′-FS1. Reaction products were resolved by gel electrophoresis and visualized by ethidium bromide staining. The amounts of radioactivity recovered from the excised gel bands were plotted against the amplification cycles (**B**) or the input RNA (**D**). M represents 100-bp molecular ladder.

intermediate between groups I and III, and follicular fluid estradiol concentrations were the highest of the three groups. Follicles of group III were the largest, and because they were obtained > 24 h after onset of estrus and their estradiol concentrations were low; presumably, they had experienced at least a portion of the preovulatory release of LH [30].

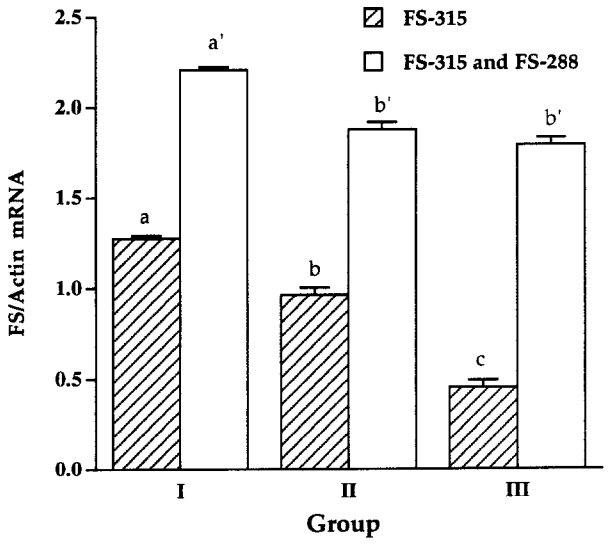

FIG. 2. Amount of DNA amplified from mRNA encoding for FS-315 and FS-315 plus FS-288 in antral follicles of gilts during advancing stages of the follicular phase of the estrous cycle; group I (early), group II (mid), and group III (late). After reverse transcription of RNA, cDNA mixtures were amplified simultaneously under the conditions given in *Materials and Methods* for 25 cycles with 3′-FS1 or 3′-FS2 primers in combination with 5′-FS that had been labeled with [γ-^{32}P]ATP. The expected bands on ethidium bromide-staining agarose gels were excised, and radioactivity of each band was determined. Amounts of DNA amplified from porcine β-actin RNA were used to normalize the results. Means differ ($p < 0.01$) within each size of amplified DNA that do not share a common superscript.

Determination of RT-PCR Conditions

Using the same strategy described previously [22], optimal PCR conditions were determined for each set of primers reported in this study. The rates of amplification were exponential between 15 and 30 cycles for both sets of primers. After 30 cycles, the rates decreased and approached a plateau at 35 cycles (see example for primers 5′-FS and 3′-FS1 in Fig. 1, A and B). On the basis of the exponential accumulation of PCR products of the FS gene with both sets of primers, 25 cycles of amplification were selected for the quantitative RT-PCR analysis. Also, aliquots of cDNA mixture equivalent to 0.0–0.4 μg total RNA were amplified for 25 cycles under the same conditions (see Fig. 1, C and D). It was found that 0.2 μg of total RNA was required to give a detectable signal on the ethidium bromide-staining agarose gels. Conditions were established as optimal for both sets of primers.

Expression of Different Forms of FS mRNA (RT-PCR)

Expression of FS-315 mRNA decreased significantly as females progressed from the early to the late stages of antral development ($p < 0.01$; Fig. 2). Follicles from group II females (n = 42) were intermediate in their mRNA concentrations relative to follicles from groups I (n = 13) and III (n = 11). Similar trends were observed for expressed concentrations of FS-315 plus FS-288 mRNAs.

To evaluate FS gene expression and follicular size, we summarized the incorporated radioactivity for small (≤ 5.0 mm), medium (> 5.0–7.0 mm) and large follicles (> 7.0 mm) within each group (Fig. 3). Expression of FS-315 and FS-315 plus FS-288 mRNAs was affected only marginally by follicular size within each group. In group II there was a small but significant increase as follicles increased from

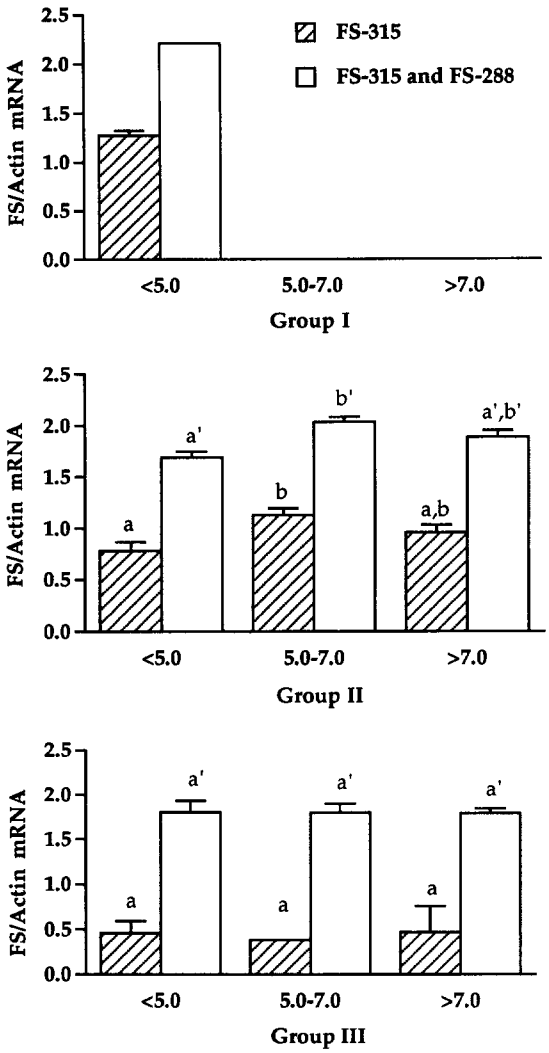

FIG. 3. Amounts of DNA amplified from mRNAs encoding for FS-315 and FS-315 plus FS-288 were plotted against follicles of small, medium, and large sizes for each group. Amounts of DNA amplified from porcine β-actin RNA were used to normalize the results. Means differ ($p < 0.05$) within each size of amplified DNA that do not share a common superscript. Numbers of follicles evaluated: for group I, 13 (≤ 5 mm); for group II, 14 (≤ 5 mm), 14 (5–7 mm), and 14 (> 7 mm); for group III, 2 (≤ 5 mm), 2 (5–7 mm), and 7 (> 7 mm).

≤ 5 mm to > 5–7 mm. In contrast, the concentration of FS-315 mRNA was 1.6 times higher ($p < 0.05$) in small follicles of group I females than in follicles of similar size in group II females. Likewise, concentration of FS-315 mRNA in large follicles of group II females was twice ($p < 0.05$) that observed in large follicles of group III females.

Expression of Different Forms of FS mRNA (RNase Protection Assay)

A schematic diagram of the assay and the expected protected fragments for FS-315 and FS-288 mRNAs are illustrated in Figure 4A. Consistent with the RT-PCR data, RNase protection assays also demonstrated that the expression levels for both FS-315 and FS-288 decreased as animals approached ovulation (Figs. 4B and 5). No significant changes in the ratio of FS-315 mRNA to FS-288 mRNA were detected among the three different follicular stages (groups) but this ratio was quite variable. As indicated in Figure 4A, two fragments (273 and 45 nucleotides) were

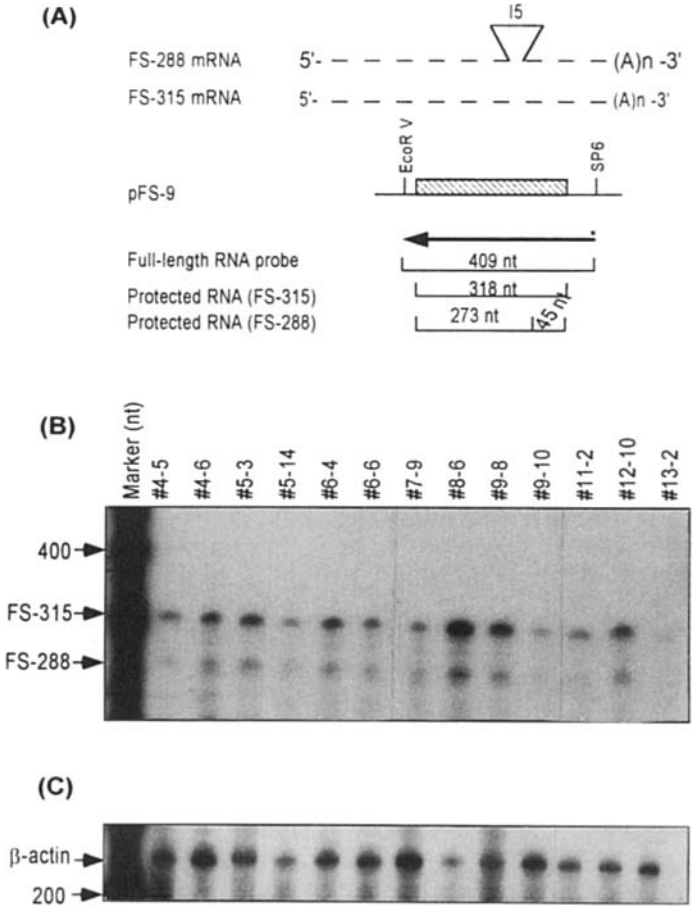

FIG. 4. RNase protection assays. **A**) The schematic diagram indicates generation of a full-length antisense RNA probe of 409 nucleotides from a *Eco*RV-linearized pFS-9 plasmid containing a partial FS-315 sequence amplified with primers 5′-FS and 3′-FS2 from cDNA that was synthesized by reverse transcription. The expected protected fragments were 318 nucleotides for FS-315 and 273 nucleotides plus 45 nucleotides for FS-288 mRNA. I5 represents unspliced intron 5 sequences in FS-288 mRNA that lead to formation of an earlier stop codon between the boundaries of exon 4 and intron 5 in FS-288 mRNA than in FS-315 mRNA. **B, C**) [^{32}P]UTP-labeled antisense pFS-9 and actin riboprobes were hybridized with RNA from 13 representative individual follicles and digested with RNase A/T1. Follicle numbers 6-4, 6-6, and 8-6 were from group I; numbers 4-5, 4-6, 5-3, 5-14, 7-9, 9-8, and 9-10 were from group II; and numbers 11-2, 12-10, and 13-2 were from group III. Mean diameters for these follicles in groups I, II, and III were 4.0, 6.1, and 8.7 mm, respectively. Data were normalized with the protected fragment for an antisense β-actin riboprobe.

protected when the antisense pFS-9 riboprobe hybridized with FS-288 mRNA, but the 45 nucleotide fragment was too small to be detected with the gel conditions used here. In contrast, no fragments were protected from RNase digestion when the sense pFS-9 riboprobe was used (data not shown), indicating that the protected fragment for the antisense pFS-9 riboprobe was specific for the FS mRNAs.

Follicular Fluid FS Concentrations

FS concentrations in follicular fluid decreased ($p < 0.01$) as females approached ovulation (Fig. 6). As expected from our scheme for classification of females, estradiol concentrations increased ($p < 0.01$) as follicles developed from the early (group I) to the mid stage (group II) and then decreased ($p < 0.01$) after the onset of estrus (group III).

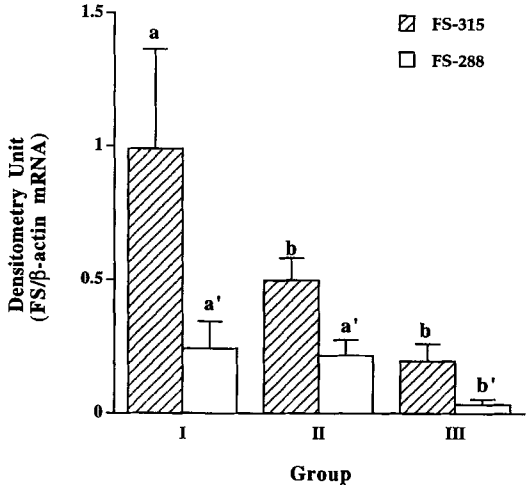

FIG. 5. Steady-state concentrations of FS mRNA in antral follicles of gilts as the follicular phase advanced from early (group I; n = 5) to mid (group II; n = 10), and late (group III; n = 9) stages. Means differ ($p < 0.05$) within each size of mRNA that do not share a common superscript.

Expression of Inhibin/Activin (I/A) Subunits (RNase Protection Assay)

RNase protection assays with the antisense riboprobes for the I/A subunits indicated that expression of α and β_A subunits increased significantly ($p < 0.05$) as follicles developed from early (group I) to mid (group II) follicular stages (Fig. 7), and then declined ($p < 0.01$) in follicles nearing ovulation (group III). No significant change was detected for the β_B mRNA between follicles of the early and mid stages. Similar to the other two I/A subunits, expression of β_B subunit decreased ($p < 0.01$) as follicles approached ovulation.

DISCUSSION

Expression of the FS gene, observed by either quantitative RT-PCR or RNase protection assays, decreased in porcine ovarian follicles during advancing stages of the follicular phase of the estrous cycle. This contrasts with earlier reports on bovine [14] and porcine follicles [31] in which FS mRNA concentrations increased with increasing follic-

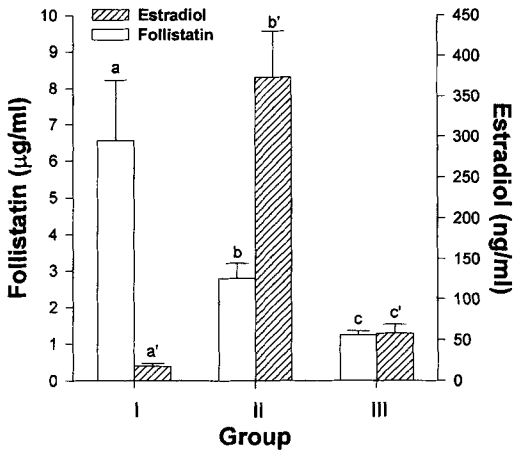

FIG. 6. Follicular fluid concentrations of FS and estradiol in antral follicles of gilts as the follicular phase advanced: group I (early), group II (mid), group III (late). Means differ ($p < 0.01$) within each hormone that do not share a common superscript.

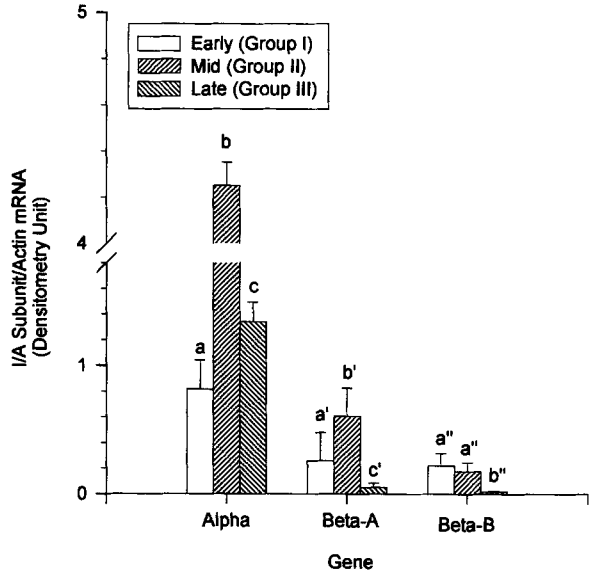

FIG. 7. Steady-state concentrations of mRNAs for I/A α, β_A, and β_B subunits in antral follicles of gilts as the follicular phase advanced: group I (early), group II (mid), and group III (late). The relative intensities of the protected fragments for these three subunits were determined by transmittance scanning densitometry and were normalized by β-actin RNA. Means differ ($p < 0.01$) within each size of mRNA that do not share a common superscript.

ular diameter. Differences in procedures for collection of follicles and stage of follicular development probably account for these differences. The previous report with porcine follicles [31] evaluated FS gene expression in follicles collected as pools of similar follicular size from prepubertal females whereas, in the present study, cluster analysis provided more homogenous groups at later, defined stages of the follicular phase. In general, we conclude that in antral porcine follicles, FS gene expression increases with follicular size during pubertal development ([31]; unpublished results) and presumably during the luteal phase of the estrous cycle; but during the follicular phase, size becomes secondary and stage of the follicular phase becomes increasingly more relevant.

Similar to the mRNA species observed in rats, cattle, and sheep, two bands of about 2.5 kb and 1.5 kb were detected from porcine follicles by Northern hybridization (data not shown), and the concentrations of these two mRNA species were relatively higher in the early- and mid-stage follicles than in follicles from the late stage. Again, this indicates that the FS gene is more highly expressed in follicles of the early follicular phase than in those of later stages. By using RT-PCR with the primers designed specifically for the FS mRNAs, we found that expression of the FS gene within an animal was less dependent on follicle diameter, within the range investigated, than on stage of the follicular phase (Fig. 3). This indicates that within groups II and III, both follicles destined for ovulation (selected follicles) and those destined for atresia did not differ markedly in FS mRNA concentrations. These results are consistent with observations in ewes and rats in which FS mRNA was detected in dominant as well as subordinate follicles, and expression decreased after the preovulatory release of LH [32, 33].

FS concentrations in the follicular fluid also decreased during the follicular phase of the estrous cycle and in general were consistent with changes observed in gene expression. FS in follicular fluid exists in multiple forms be-

cause of proteolytic cleavage of FS-315 to FS-303 and because of various forms of glycosylation of FS-315, FS-303, and FS-288 [34]. These truncated forms of FS bind to heparan sulfate proteoglycans on cells [35]; thus, concentrations within follicular fluid probably differ somewhat from those within the granulosa cell layer. During the early stages of the follicular phase, follicular fluid FS concentrations were higher than those observed later in larger, estrogen-active follicles from females during the mid stage of the follicular phase. The concentration of FS decreased more dramatically in follicular fluid than did that of its mRNA in follicular tissue. This may reflect differences in the two separate populations of females used in these studies or, more likely, differences in tissue compartmentalization. FS binds activin with an affinity similar to that of activin receptors, and FS neutralizes the effects of activin [9, 35, 36]; therefore, the role of FS in porcine antral follicles may be to retard access of activin to granulosa cells. When granulosa cells from porcine antral follicles were obtained at different stages of follicular development, activin had inhibitory effects on both estradiol and progesterone production [37, 38]. As a consequence, FS may partially protect granulosa cells from the atretogenic action of activin during early antral development, but this protection becomes of diminishing importance as follicles experience the ovulatory release of gonadotropins and steroidogenesis declines. This prediction is consistent with observations in rats where FS was detected only in dominant preovulatory follicles on proestrus but decreased after the preovulatory release of gonadotropins [32, 39].

Our RNase protection results on the expression of I/A α and β_A subunits in porcine follicles confirmed observations reported by Guthrie et al. [18] and extend these to include β_B subunit. Expression of β_B subunit mRNA was much lower than that for the α and β_A subunit transcripts, and this expression decreased as follicles progressed from the mid to the late stage of the follicular phase. Expression of both I/A α and β_A subunits increased significantly (ca. 2–5-fold) as follicles developed into the mid stage. Then, as follicles approached ovulation and granulosa cells became more differentiated, expression of all three subunits decreased markedly in concert with follicular fluid estradiol concentrations. These changes in mRNA concentrations mimic changes observed in plasma immunoreactive inhibin concentrations during the follicular phase [40].

Cluster analysis provided a technique for grouping animals that relies on information from multiple variables associated with follicular development. The objective of cluster analysis is to look for groups in which all subjects in a group are relatively "similar" to each other but relatively "different" from all subjects in other groups. With this multivariate analytical technique, we used morphological and biochemical characteristics of the follicles from each female. This technique provided more homogenous groups than attainable by assigning females on the basis of days from their previous estrus.

In summary, we demonstrated with RT-PCR and RNase protection techniques that the FS gene is more highly expressed in follicles during early than during late stages of the follicular phase. This pattern of expression was related more to the animal's stage of the follicular phase than to follicle size itself, and this pattern differed from that observed for the I/A subunits. No significant differences were detected in the ratio of mRNA for FS-315 to that for FS-288 among follicles at the three different stages of the follicular phase. In accordance with changes in gene expression, concentrations of FS in follicular fluid decreased during the final stages of follicular development. FS binds activin with high affinity, but how these two proteins interact in vivo to modulate antral follicular development is not understood. Increased concentrations of FS within developing follicles should protect these follicles from the inhibition of steroidogenesis by activin that has been observed in cultured, highly differentiated, granulosa cells [38, 41–43].

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