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Unilateral Ovariectomy Increases Inhibin Messenger Ribonucleic Acid Levels in Newly Recruited Follicles*

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ABSTRACT. We have used the unilaterally ovariectomized (ULO) rat as an experimental model to assess transient relationships between follicular recruitment and inhibin mRNA levels in the ovary. Animals were divided into three experimental groups (ULO, sham-operated, and intact) and killed 0, 9, or 24 h postsurgery. Inhibin α- and β_A mRNA were measured within individual ovarian follicles by *in situ* hybridization. Serum FSH concentrations in ULO animals increased significantly at 9 h and returned to presurgery levels by 24 h. There was no effect of surgery on serum LH concentrations. In the ULO animals, compensatory follicular recruitment was observed in the remaining ovary at 24 h as a significant increase in the number of follicles with mean diameter greater than 350 μm. Two distinct populations of healthy maturing follicles, based on diameter and hybridization intensity, were observed in ovaries from 24 h post-ULO rats. The first class, NRF-1 (newly recruited follicle-1), were those recruited 48 h previously by the secondary FSH surge of the preceding cycle. These large follicles (>500 μm mean diameter) hybridized to both inhibin probes. The second class of follicles (NRF-2), which represent those newly recruited by the ULO-induced increase in serum FSH, were smaller in size than NRF-1 (350–500 μm mean diameter). The intensity of hybridization of both inhibin probes to NRF-1 was higher than that to NRF-2 in the majority of follicles. Each NRF-1 contained higher levels of both α- and β_A-inhibin mRNA than nonrecruited follicles (150–350 μm diameter). These data suggest that subtle changes in serum FSH concentrations, which are sufficient to induce follicular recruitment into a size class capable of ovulating within a given cycle, play the additional role of enhancing inhibin mRNA levels in developing follicles (*Endocrinology* **124:** 310–317, 1989)

THE OVARIAN hormone inhibin regulates secretion of pituitary FSH and, as a consequence, performs a key role in maintaining the female reproductive cycle. Inhibin has been isolated and characterized as an α-β heterodimer (1–4), and two closely related forms of the β-chain (β_A and β_B) have been identified (1, 5). cDNAs for the inhibin α- and β-subunits have been sequenced from a variety of mammalian species (5–9). Although studies have focused on the structure of inhibin as well as the interaction between inhibin and the gonadotropins (10–12), regulatory mechanisms controlling synthesis of this ovarian hormone have yet to be completely elucidated.

Levels of both inhibin (13) and its mRNA (14) vary during the course of the 4-day estrous cycle in the rat. We have previously demonstrated a progressive increase during the early portion of the rat estrous cycle in the amount of inhibin α- and β_A mRNA present in ovarian follicles (14). Peak mRNA levels are attained by the afternoon of proestrus and then decline rapidly after the primary LH and FSH surges. During the early morning of estrus inhibin mRNA is detectable in newly recruited follicles (350–500 μm).

The recruitment of a new cohort of ovarian follicles with the potential to ovulate during the next cycle has been shown to be triggered by the secondary surge of FSH in the presence of sufficient LH (15, 16). We propose that these elevated levels of FSH, which are maintained through the morning of estrus, may have the additional function of increasing the production of inhibin mRNA in these new recruits. The return of serum concentrations of FSH to basal values during the morning of estrus would be the natural consequence of translation of the inhibin mRNA and secretion of inhibin.

We have used the unilaterally ovariectomized (ULO) rat as an experimental model to test whether FSH-induced recruitment of ovarian follicles, independent of cycle stage, is accompanied by increased follicular content of inhibin mRNA. In the ULO rat, serum FSH concentrations are transiently elevated in the absence of a detectable effect on serum LH or estradiol (17–19). Twenty-four hours after ULO, the number of newly recruited follicles in the remaining ovary doubles, while

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serum FSH concentrations return to control values (17–20). Using this model we were able to assess whether a transient rise in serum FSH levels, sufficient to increase follicular recruitment, has the additional function of augmenting inhibin mRNA levels in the rat ovary.

Materials and Methods

Animals

Sixty-day-old female Sprague-Dawley CD rats (Charles River, Portage, MI) were housed in temperature-controlled quarters on a 14-h light, 10-h dark cycle, with lights on at 0500 h. Animals were provided with food and water *ad libitum*. Cycle stage was assessed by daily inspection of vaginal cytology, and only those animals showing two consecutive 4-day estrous cycles were used.

Surgery and collection of tissue

Animals were divided into three experimental groups: ULO, sham-operated (sham), and no surgery (intact). Surgery was performed at 0900 h on metestrus under ether anesthesia. Nine and 24 h after surgery, animals from each experimental group were decapitated: two intacts at both 9 and 24 h, three shams at both 9 and 24 h, and four ULOs at both 9 and 24 h. In addition, three intacts were killed at 0900 h on metestrus.

Trunk blood was collected and allowed to clot overnight at 4 C. After centrifugation, sera were aliquoted and stored at −20 C for RIA. Ovaries with attached oviduct were immediately excised, frozen on dry ice, and stored at −70 C for subsequent *in situ* hybridization analysis.

In situ hybridization

Ovaries were oriented with respect to their attached oviduct and sectioned (20 μm) using a Reichert (Buffalo, NY) 840 cryostat. Approximately 100 sections were obtained from each ovary and mounted alternately on 2 microscope slides so that adjacent sections could be hybridized to either the α- or β_A-inhibin probes (8, 14). Slides were then fixed in 5% paraformaldehyde at room temperature for 5 min. Hybridization was performed with ^{32}P-labeled antisense inhibin α (5′ *Pst*I to *Eco*RI fragment from rINA-13; 570 basepairs)- and β_A (5′ *Pst*I to *Eco*RI fragment from rINB-5; 514 basepairs)-subunit riboprobes using conditions previously described (8, 14). Controls included hybridization of ^{32}P-labeled antisense probes to liver sections and hybridization of sense-strand inhibin probes to ovary. Slides were exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY) for approximately 10 h at room temperature. Autoradiography was also performed directly on tissue sections using Kodak NTB-2 emulsion, and slides were developed after a 15-day exposure at 4 C. Tissue sections were then stained with hematoxylin and eosin. Microphotography was performed on a Nikon Optiphot microscope.

Follicular diameter was determined in follicles using a calibrated ocular micrometer. For each follicle, measurements were taken in a section in which the oocyte was clearly visible. A maximum diameter and a diameter at right angles to it were used to calculate mean diameter. Follicles were then classified according to size. Ovarian follicles from rats 24 h after surgery were divided into three separate classes based on mean follicular diameter (Table 1): 1) NRF-1 (newly recruited follicle-1) included follicles more than 500 μm in diameter; these follicles were new recruits 24 h before surgery; 2) NRF-2 included follicles in the size range 350–500 μm, which is the size previously reported for new recruits (15, 21); and 3) small nonrecruited follicles (SMF) included small follicles with diameters ranging from 150–350 μm.

In the present study all newly recruited follicles hybridized to both the α- and β_A-inhibin probes. As a consequence, we were able to determine the number of new recruits in each ovary by visual inspection of autoradiographic films and counting follicles that hybridized to both inhibin probes. Although each hybridizing follicle appeared in more than 1 20-μm ovarian section, it was only counted once. Since the actual number of sections recovered from each ovary varied, the number of hybridizing follicles was standardized per 100 sections for each animal and is reported as the number of hybridizing follicles per 100 sections.

Hormone RIAs

Serum was assayed for LH by the ovine-ovine RIA using NIH LH S16 as standard and S-15 antiovine LH antibody. Serum FSH concentrations were measured using the rat-rat NIDDK system with FSH RP-1 as standard. For both LH and FSH, serum samples were measured in duplicate within the same RIA. Intraassay coefficients of variation for low and high serum pools were 2% and 1.5%, and 11% and 8% for LH and FSH, respectively.

Statistics

All values are reported as the mean ± SE. Data for the 9 and 24 h postsurgery groups were analyzed in a two-way analysis of variance (ANOVA), with the two between-treatment factors being surgery (intact *vs.* sham *vs.* ULO) and autopsy time (9 *vs.* 24 h). Data for intact animals were also analyzed in a one-way ANOVA, with the between-group factor being autopsy time (0 *vs.* 9 *vs.* 24 h). All statistics were computed using CRISP (Crunch Interactive Statistical Package, Crunch Software, San Francisco, CA).

Results

Serum concentrations of LH and FSH for all experimental groups are shown in Fig. 1. As expected, ULO resulted in an increase in serum FSH concentrations 9 h after surgery (Fig. 1A). FSH had returned to control levels by 24 h post surgery. Two-way ANOVA indicated that serum FSH concentrations were significantly affected by autopsy time; *post-hoc* testing revealed that levels in the ULO animals were significantly higher than those in both intacts and shams, as Fig. 1 suggests. Serum LH concentrations were similar in the intact, sham, and ULO rats at each time point, with no effect of surgery (Fig. 1B). There was, however, a significant effect of autopsy time, due to the lower LH values in all experimental groups 24 h after surgery. The reason for this decrease in serum LH is not readily apparent, but could

TABLE 1. Follicle classes 24 h postsurgery in ULO rats

Class	Mean diameter (μm)	Inhibin mRNA expression		Present in	Interpretation
		α	β_A		
NRF-1	>500	+++[a]	+++[a]	All ovaries	Recruited 48 h earlier by secondary FSH surge
NRF-2	350–500	++	++	ULO ovaries only	Recruited 24 h earlier by ULO
SMF	150–350	+	−	All ovaries	Prerecruitment for this cycle

[a] ++ for a small number of follicles.

be due to a normal small decrease in LH between metestrus and diestrus.

To assess changes in both the number of ovarian follicles producing inhibin mRNA and the relative amount of inhibin mRNA per follicle, *in situ* hybridization analysis was used. The number of follicles hybridizing to both the α- and β_A-inhibin probes was determined for each experimental group and is shown in Fig. 1C. At both 0 and 9 h, the number of hybridizing follicles was similar in all experimental groups (Fig. 1C). Twenty-four hours after ULO, however, there was a significant increase in the number of hybridizing follicles relative to those in both intact and sham controls (Figs. 1C and 2).

Nine hours after surgery (1700 h on metestrus), hybridization to both inhibin probes was detected in all healthy follicles that had been recruited on the morning of estrus (NRF-1; data not shown). The intensity of hybridization to either the α- or β_A-inhibin probes (which reflects the amount of mRNA present) was similar in NRF-1 from intact, sham, and ULO animals at this time point.

Twenty-four hours after ULO, two distinct populations of follicles were distinguishable. The first class of follicles (NRF-1) were those previously recruited by the secondary FSH surge of the preceding cycle (24 h before surgery). These follicles (>500 μm mean diameter) hybridized to both the α- and β_A-inhibin probes (Fig. 2) and were present in ovaries from both ULO (Figs. 2, A–C, and 3, A–C) and control animals (Figs. 2, D–F, and 3, G–I). The second class of follicles (NRF 2) were those newly recruited by the ULO-induced increase in serum FSH (Figs. 2, A–C, and 3, D–F). These smaller follicles (350–500 μm mean diameter) were numerous in ovaries from ULO animals, but not in sham-operated or intact controls. Importantly, each follicle in this size class showed hybridization to both the α- and β_A probes. The intensity of hybridization of both inhibin probes to NRF-1 was higher than that to NRF-2 in the majority of follicles (Fig. 3, B–C, E, and F). A small number of NRF-1, however, showed hybridization similar in intensity to that of NRF-2 indicating some overlap in the range of intensity.

In addition to the two populations of recruited follicles (NRF-1 and NRF-2) present 24 h after ULO, another follicle population was present in ovaries from all experimental groups, regardless of treatment. These SMF hybridized to the α-inhibin probe, but at lower levels than NRF-2 (Fig. 4, A and B). There was no detectable hybridization above background to the β_A-inhibin probe in SMF (Fig. 4C), in contrast to the obvious hybridization seen in NRF-2 (Fig. 4D). Based on their small size (150–350 μm mean diameter) these follicles would not be expected to ovulate during the cycle in progress (22). We have summarized the findings on the three size classes of follicles (NRF-1, NRF-2, and SMF) in Table 1.

The transient rise in serum FSH that followed ULO (Fig. 1A) consistently increased the amount of inhibin α- and β_A mRNA in NRF-2 relative to that in SMF (Fig. 4). The effect of these experimentally elevated FSH concentrations on inhibin mRNA levels in previously recruited follicles (NRF-1), however, was not as clear. Although hybridization intensity appeared to be enhanced in most NRF-1 that had been exposed to the increased FSH concentrations (ULO-24 h; Figs. 2, B and C, and 3B) relative to those that had not (sham-24 h; Figs. 2, E and F, and 3E), this result was not consistent in all follicles.

Discussion

We have used the ULO rat as a model to assess the interplay between ovarian follicular recruitment and the expression of inhibin mRNAs in the follicle. Consistent with previous studies (17–20), ULO resulted in a significant increase in serum FSH levels, with no detectable change in serum LH concentrations. Follicular recruitment was observed at 24 h as a significant increase in the number of ovarian follicles with mean diameter greater than 350 μm. Importantly, each healthy NRF displayed enhanced levels of both inhibin α- and β_A-subunit mRNAs relative to those in unrecruited follicles. These results suggest that subtle and transient changes in serum FSH concentrations can be important in regulating inhibin mRNA in developing follicles.

Although the phenomenon of compensatory follicular recruitment in ULO rats has been well documented (17, 18, 20), the mechanism for the decline in serum FSH concentrations that occurs by 24 h post-ULO has not been elucidated. The FSH-induced increase in inhibin mRNAs after ULO would most likely result in elevated serum inhibin levels, which would further inhibit FSH secretion from the pituitary. The end result should be the restoration of presurgery serum FSH concentrations.

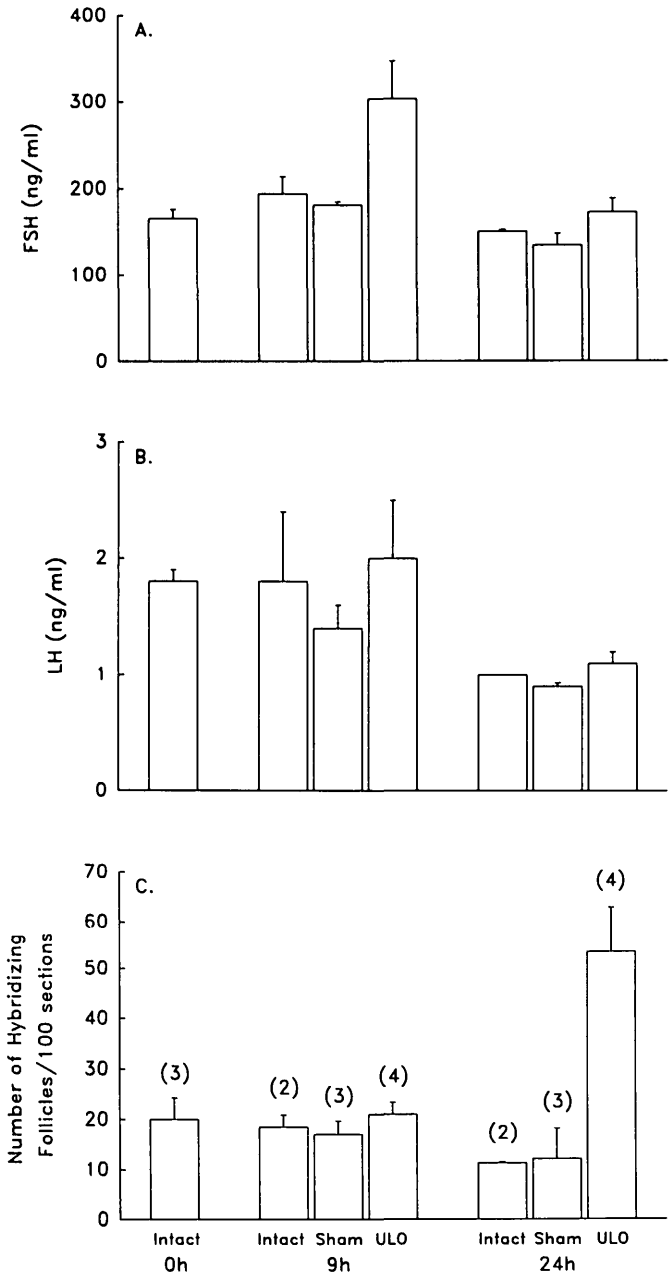

FIG. 1. Serum concentrations of FSH and LH and number of ovarian follicles hybridizing to both α- and β_A-inhibin subunit probes in intact, sham, and ULO rats. Animals were decapitated 0, 9, and 24 h after surgery, as indicated. Values are given as the mean ± SE. *Numbers in parentheses* in C indicate the number of animals in each group. By two-way analyses of variance, FSH-autopsy time, $P(1,17) = 0.02$; Newman-Keuls *post-hoc* analysis, ULO > sham and intact, $P < 0.01$; LH-autopsy time, $P(1,17) = 0.03$; number of hybridizing follicles-surgery, $P(2,16) < 0.05$.

Whereas the inhibin secretion theory has been previously hypothesized to account for the decrease in serum FSH by 24 h post-ULO (23), the present results provide evidence for changing inhibin mRNA levels in this experimental model.

Two classes of healthy maturing follicles were observed 24 h after ULO. The most intense hybridization was detected in the largest class of follicles which had been previously recruited by the secondary FSH surge approximately 24 h before the beginning of the study (NRF-1). A second class included smaller follicles recruited as a result of ULO (NRF-2). Hybridization intensity was higher in NRF-1 than in NRF-2 in the majority of follicles. In a small number of NRF-1, however, there was some overlap in the hybridization intensity with the smaller size class. Both NRF-1 and NRF-2 have the capacity to ovulate in that given cycle; the net result being a doubling of the number of ovulations from the remaining ovary by the next estrus (24).

The increased amount of inhibin mRNA present in most NRF-1 relative to that in NRF-2 is similar to the progressive increase in inhibin mRNA that occurs during the normal rat estrous cycle (14). Growing follicles are exposed to ambient low levels of both LH and FSH during the cycle (25) which are probably responsible for the increased inhibin mRNA levels observed. In the present study NRF-1 had been exposed to the gonadotropins approximately 24 h longer than NRF-2 after recruitment, which could account for the enhanced levels of inhibin mRNA in these previously recruited follicles (NRF-1). The range in hybridization intensity observed in NRF-1 may reflect variations in developmental maturity which result in differential responsiveness to circulating gonadotropins.

In addition to developing follicles which contained both α- and β_A-inhibin mRNAs, we observed smaller follicles (<350 μm), which we called SMF. These nonrecruited follicles hybridized to the α probe, but showed no hybridization above background levels to the β_A probe. This observation suggests a possible differential regulation of the inhibin genes before recruitment which allows for continuous expression of α mRNA at low levels in antral follicles. This finding helps to clarify why levels of inhibin α- and β_A mRNA are similar in preovulatory follicles by *in situ* hybridization (14), yet in total ovarian RNA the α-subunit is detected in 6- to 10-fold excess over β_A (8, 9, 26). In addition, it has been observed in granulosa cell cultures, that the α-subunit is expressed and the translation product stored until expression of the β_A-subunit (27). The results of the present study are consistent with these observations and further suggest that the β-subunit is only expressed when the ovarian follicle is committed to the ovulatory pool by FSH induction.

Regulation of the female reproductive cycle involves the interaction of peptide and steroid hormones originating in the hypothalamus, anterior pituitary, adrenals, and ovaries. Inhibin, through its feedback control of FSH secretion, plays an important role in the integration of this system. We have previously demonstrated that inhibin mRNA in preovulatory follicles increases progressively during the early part of the estrous cycle and

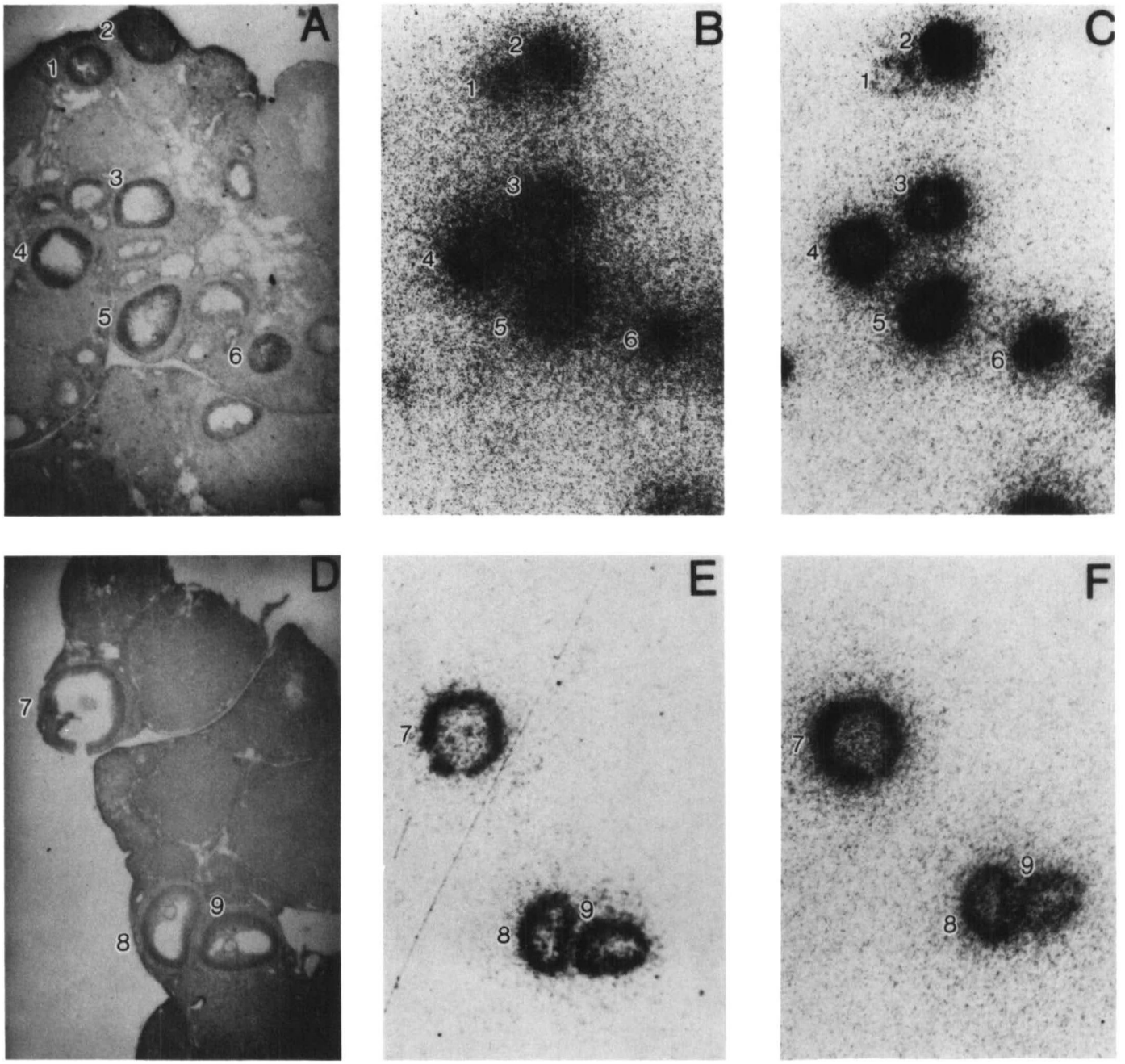

FIG. 2. Histology and hybridization of ovarian sections from ULO and sham rats 24 h after surgery. A and D show ×25 magnification of ovarian cross-sections from a ULO and a sham rat, respectively. B and E show corresponding autoradiographs after *in situ* hybridization to the α-inhibin probe, and C and F after hybridization to the β_A probe. Hybridizing follicles in A and D are numbered, and corresponding signals on autoradiographs are identified with the appropriate follicle number. Mean diameter for hybidizing follicles is listed below:

Follicle no.	Type	Mean diameter (μm)
1	NRF-2	416
2	NRF-1	Not measured
3	NRF-2	458
4	NRF-1	525
5	NRF-1	530
6	NRF-2	440
7	NRF-1	730
8	NRF-1	530
9	NRF-1	582

Follicles more than 350 μm in mean diameter which showed no detectable hybridization to either inhibin probe were clearly atretic and are unnumbered in A.

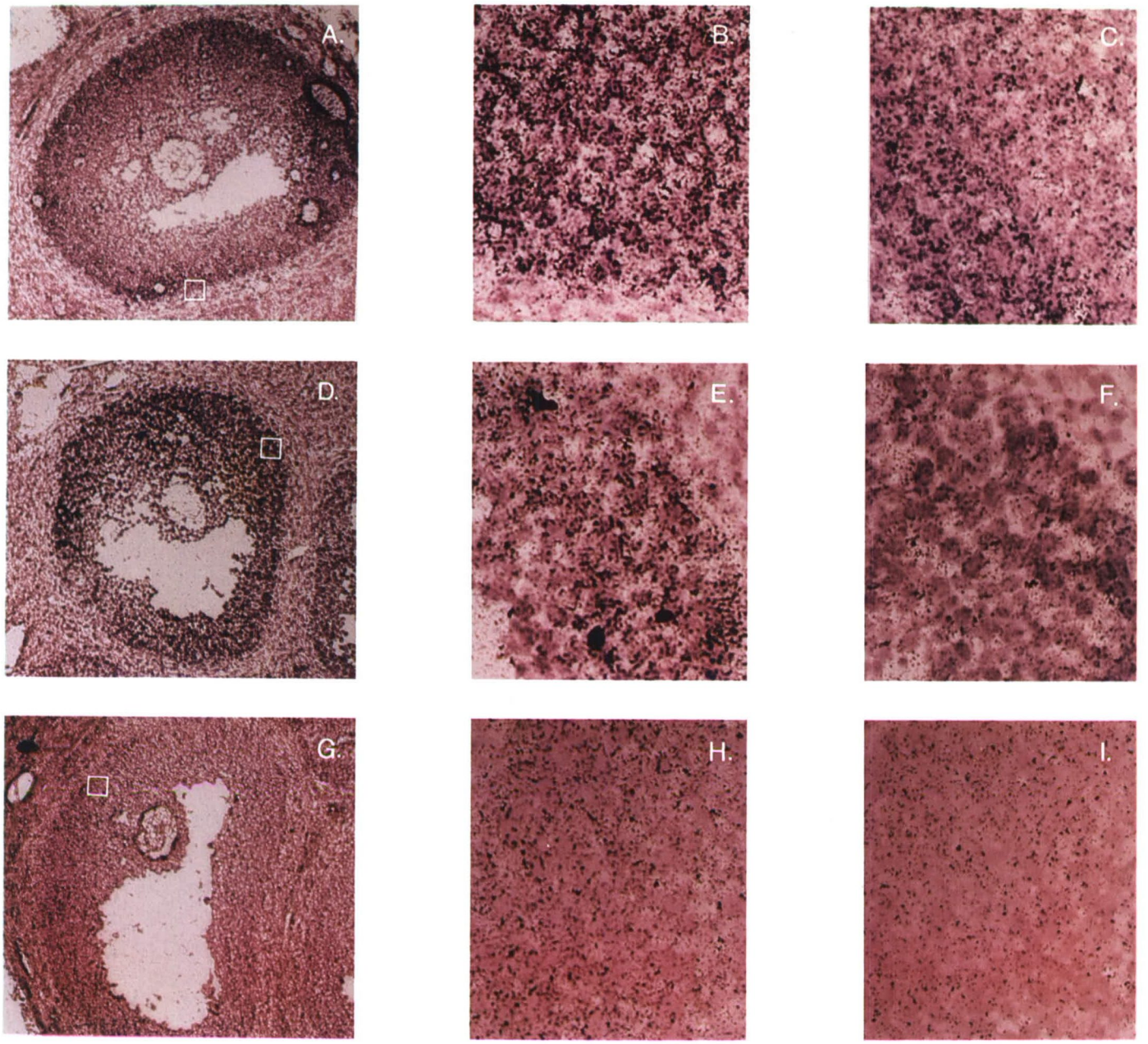

FIG. 3. *In situ* hybridization analysis of inhibin mRNAs in ovaries from ULO and sham rats. A, D, and G show cross-sections of ovarian follicles (×100 magnification) from ULO (A and D) and sham-operated (G) rats 24 h after surgery (A, NRF-1; D, NRF-2; G, NRF-1). B, E, and H (α probe hybridization) and C, F, and I (β_A probe hybridization) were photographed at ×500 magnification to allow visualization of individual silver grains within the granulosa cells of follicles shown in A, D, and G. Mean follicular diameters: A, 566 μm; D, 458 μm; G, 582 μm.

attains maximum levels on the afternoon of proestrus (14). During late proestrus, mRNA levels for inhibin decrease dramatically in response to the primary gonadotropin surges (our manuscript submitted). This decline in inhibin mRNA production presumably reduces circulating inhibin, which permits the prolonged secretion of FSH during late proestrus and early estrus. Not until the early morning of estrus do inhibin mRNA levels increase, this time in follicles newly recruited into the next cycle in response to the secondary FSH surge. It seems apparent from the present study that one cue for enhanced production of inhibin mRNA in these new recruits is FSH itself.

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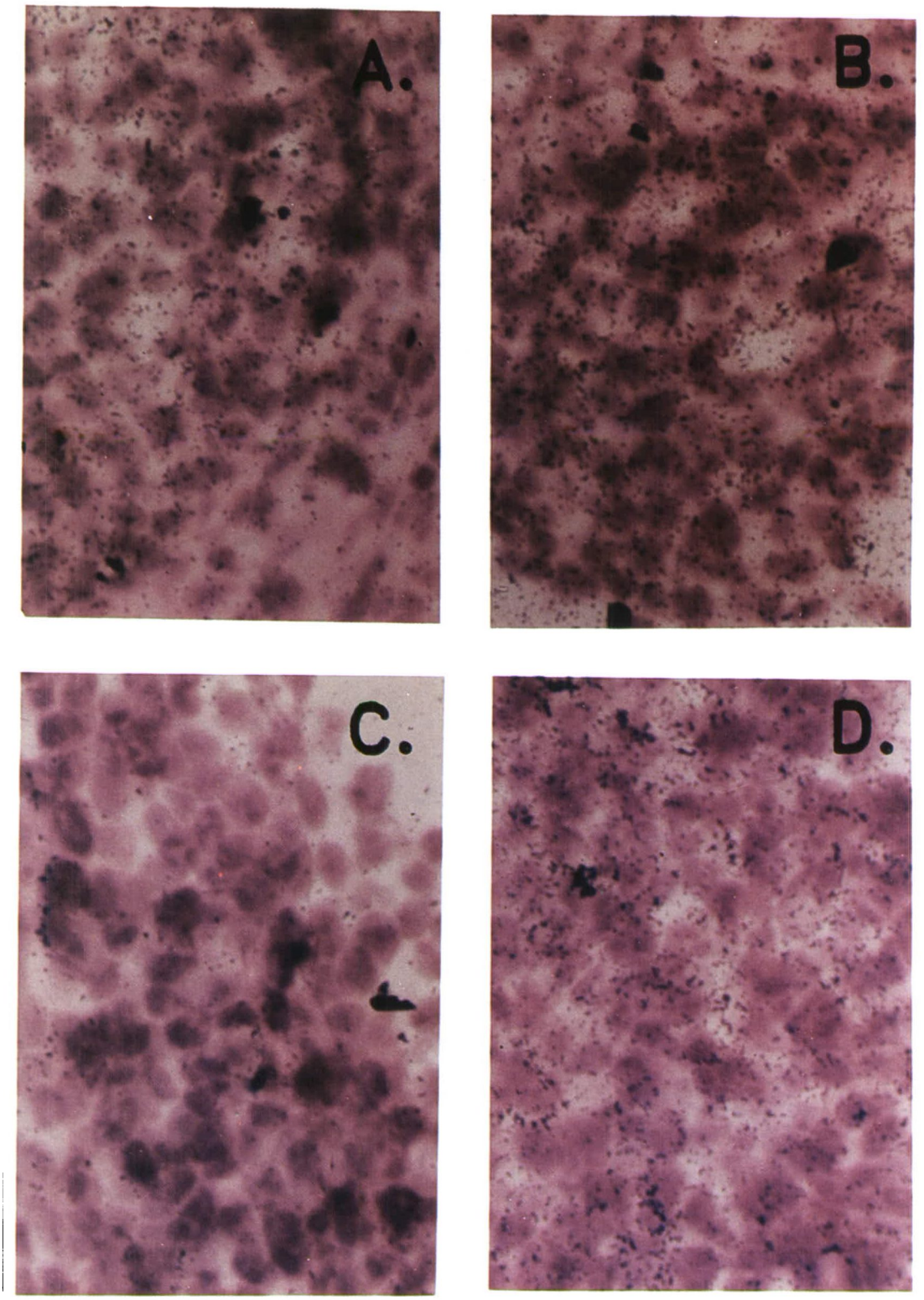

FIG. 4. Hybridization of inhibin α- and β_A-subunit probes to SMF and NRF-2. Ovary is from an animal killed 24 h after ULO. A and B show hybridization of α-inhibin probe to cross-section of SMF (242 μm mean diameter) and NRF-2 (386 μm mean diameter), respectively. C and D show hybridization of these same follicles to β_A-inhibin probe. Magnification, ×500.

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