0013-7227/02/$15.00/0
*Printed in U.S.A.*

Endocrinology 143(6):2385–2398


# Expression of ER-α and ER-β in the Hamster Ovary: Differential Regulation by Gonadotropins and Ovarian Steroid Hormones

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**Spatiotemporal expression patterns of ER-α and ER-β protein and mRNA in hamster ovarian cells during the estrous cycle and following hypophysectomy and selective hormone replacement were evaluated by immunofluorescence, immunoblotting and *in situ* hybridization analyses. Whereas ER-β mRNA and protein expression predominated in granulosa cells and ER-α expression was in interstitial and thecal cells, overlap in receptor subtype expression across cell types was evident. Both ER subtypes were present from primordial follicle stage onward. ER-α mRNA levels and immunoreactivity started increasing from D3:0900 h in intersitial and granulosa cells and peaked on the proestrous (D4:0900 h). Regionalized higher expression of ER-α in granulosa cells in and around the forming antrum was evident. Surface epithelial cells were also positive. ER-β mRNA and protein expression increased markedly in granulosa and interstitial cells on D2:0900 h, reached a peak on D3:0900 h, and then declined sharply on D4:0900 h. No change in ER expression occurred following the preovulatory gonadotropin surge. Whereas FSH or human CG stimulated ER-α mRNA and protein expression in hypophysectomized hamsters, only FSH could stimulate ER-β mRNA and protein, and the effect was significantly attenuated by human CG. ER expression was stimulated by estrogen, but progesterone strongly inhibited estrogen action. These results indicate that ER expression is cell type specific to the larger extent and is critically regulated by reproductive hormones. (*Endocrinology* 143: 2385–2398, 2002)**

ESTROGENS PLAY AN important role in the development and functioning of female reproductive tissue (1–3), including ovarian granulosa cells (4) via its cognate nuclear receptors (5–7). Estrogen receptor belongs to the nuclear receptor superfamily (8) that includes estrogens, progestins, androgens, adrenal steroids, thyroid hormone, and vitamin D3 (7, 9). Autoradiographic, immunocytochemical, and radio-receptor analyses have provided evidence for the presence of ERs in brain, pituitary, gonads, and reproductive tracts (3, 10–14). Further, radioligand studies have demonstrated the presence of specific estrogen binding to granulosa cells of rats (4, 15, 16), mice (17), rabbits (17), guinea pigs (17), and hamsters (3, 16–20). These lines of evidence clearly indicate that ovarian granulosa cells are a major site of estrogen action and form the foundation for the discovery of estrogen receptor subtypes in ovarian cells.

Most of the earlier studies on ER have focused on the classical ER (3), ER-α, which was cloned by Green *et al.* (21); however, a second ER, ER-β, has been cloned from the rat prostate (22) and mouse ovary (23). Subsequently, ER-β has been cloned from tissues from a variety of species, including human (24–28). Although, both ER-α and ER-β bind estrogen with similar affinity (29), ER-β shows higher affinity to many estrogenic compounds (29). Using immunohistochemistry and *in situ* hybridization techniques, protein and mRNA of ER-α and ER-β have been primarily localized in thecal and stromal cells and granulosa cells, respectively, of rat (3, 22, 30–36), mouse (37), cow (38), monkey (39–41), and human (25, 42). Besides this cell type-specific localization, evidence has accumulated for the dual presence of ER subtypes in rat (32, 36), monkey (41), and human (42, 43) granulosa, theca, and interstitial cells, although the evidence is more consistent for primate and human ovarian cells (3). However, the relative distribution of both receptor subtypes in any given cell type is not so clear. Sharma *et al.* (44) have shown that small amount of immunoprecipitatable ER-α is present in rat granulosa cells and both Forskolin and E2 increase the receptor up-regulation as determined by immunofluorescence studies. Ovaries of ER-α knockout (αERKO) mice contain hemorrhagic and cystic antral follicles (45), the major cause of which has been ascribed to higher levels of serum LH (45–47); however, a low rat of ovulation can be induced by exogenous gonadotropins (45). Although some antral follicles develop in βERKO mice, these are significantly deficient in granulosa cells and respond very poorly to gonadotropins (47–49). On the other hand, mice with ER-αβ double deletion have severe abnormalities in folliculogenesis and do not ovulate in response to exogenous gonadotropins (49). All these lines of evidence indicate that species-specific differences in the expression patterns of ER exists in ovarian cells, which may lead to differential effect of estrogen across ovarian cell types. Although the presence of estrogen binding has been documented in the hamster ovary (17, 20), cell type-specific spatiotemporal expression of ER subtypes in the hamster ovary has not yet been evaluated. The objectives of the present study were to partially clone hamster ER-α and ER-β, systematically evaluate the spatiotemporal expression patterns of ER-α and ER-β protein and mRNA in hamster

Abbreviations: ERE, Estrogen response element; ERKO, ER knockout; hCG, human CG; Hx, hypophysectomized; P, progesterone; SSC, saline sodium citrate.

ovarian cells under endogenous hormonal milieu throughout the estrous cycle, and delineate the role of gonadotropins and ovarian steroid hormones in regulating ER subtype expression in ovarian cells by selective hormone replacement.

## Materials and Methods

Antibodies to ER-α and ER-β were from Novacastra Laboratories Ltd. (NewCastle, UK) and Upstate Biotechnology, Inc. (Lake Placid, NY), respectively. Antiaromatase antibody was a gift from Dr. D. C. Johnson (University of Kansas Medical Center, Kansas City, KS). Alexa-conjugated second antibodies were from Molecular Probes, Inc. (Eugene, OR); second antibodies for Western blotting chemiluminescence were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); WestFemto chemiluminescence substrate was from Pierce Chemical Co. (Rockford, IL); Optitran transfer membrane was from Schleicher & Schuell, Inc. (Dassel, Germany); PCR chemicals were from Roche Molecular Biochemicals (Indianapolis, IN), Amersham Pharmacia Biotech Boehringer (Piscataway, NJ), and Promega Corp. (Madison, WI); TOPO PCR cloning kit was from Invitrogen (Carlsbad, CA); [$^{35}$S]-α-CTP (specific activity, 800 Ci/mmol) was from ICN Radiochemicals (Costa Mesa, CA); and riboprobe synthesis kit was from Promega Corp. All other molecular-grade chemicals were purchased from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), or United States Biochemical (Cleveland, OH). Ovine-FSH-20 was purchased from the National Pituitary Hormone Program (NIDDK, NIH, Bethesda, MD).

Female golden hamsters (90–100 g, Sasco, Kingston, NY) with three consecutive estrous cycles were housed under controlled climate, and 14-h light/10-h dark cycle according to the U.S. Department of Agriculture and Institutional Animal Care and Use Committee (IACUC) guidelines. The use of hamsters in this study was approved by the IACUC. Ovaries were obtained at 0900 h of each day of the estrous cycle and also at 1600 h on proestrous (D4) following the periovulatory gonadotropin surge, and snap frozen on dry ice. To obtain ovaries from hypophysectomized (Hx) hamsters, females were hypophysectomized on d 1:0900 h (estrous) as previously described (50). Ten days after Hx, hamsters was injected sc with (1) 10 μg ovine-FSH-20 twice daily for 2 d, (2) a single dose of 10 IU human CG (hCG) (Sigma), or (3) a combination of FSH (twice daily for 2 d) and hCG (single injection on the first day, different site) in 0.5% BSA in saline. Because the *in vivo* half-life of hCG is 72 h, it would be expected to interact with LH receptors whenever present during the study period. Control animals received equal volume of vehicle. Ovaries were collected 48 h after the first injection. The second group of Hx hamsters was injected sc with a single dose of 1) 0.1 mg of E2-valerate (Pharmacia-Upjohn Co., Kalamazoo, MI), 2) 0.5 mg progesterone (Steraloids, Wilton, NH) in sesame oil (Sigma), 3) a single E2 injection followed 6 h later by a single dose of progesterone, or 4) equal volume of sesame oil vehicle. Ovaries were collected 24 h after the injection. Ovaries from all groups were snap frozen on dry ice.

### *Immunofluorescence detection of ER-α or ER-β*

Ovaries were sectioned at 7 μm in a Leica cryostat (North Central Instruments, Minneapolis, MN) at −18 C. To detect ER-α protein, sections were fixed 10 min in Zamboni's fixative (51) at room temperature, whereas for ER-β, sections were fixed sequentially in ice-cold methanol for 3 min, ice-cold ethanol for 3 min, and 4% freshly made paraformaldehyde in PBS (pH 7.4) for 10 min at room temperature, followed by three 5-min washes in PBS at room temperature (52). After blocking nonspecific protein-binding sites with PBS containing 10% donkey serum, 0.1% Triton X-100, and 0.1% sodium azide for 30 min at room temperature (ER-α) or 1 h at 4 C (ER-β) in a humidified chamber, sections were exposed overnight to optimal dilution of the receptor-specific antibody at 4 C in a humidified chamber. Sections were then rinsed twice for 5 min each in PBS, followed by 30-min exposure to appropriate second antibody conjugated with Alexa-488 (green fluorescence) in a humidified chamber at room temperature. Nuclei were stained simultaneously with propidium iodide (red contrast for Alexa-488). After thorough rinsing, sections were mounted with Fluoromount G (Southern Biotechnology Associates, Inc., Birmingham, AL) and evaluated under epifluorescence in a Leica Corp. DMR research microscope equipped with an Optronics Magnafire digital camera (Optronics, Goleta, CA).

To verify the specificity of the antibody recognition of receptor subtypes, sections of ovaries were incubated with 10% donkey serum or antibodies preabsorbed to 100-fold excess of recombinant human ER-α (Panvera, Madison, WI) or a control ER-β peptide (Upstate Biotechnology, Inc.) and processed identically as mentioned earlier.

For digital image capturing, the exposure time was adjusted using sections incubated without the primary antibody to subtract any auto- or nonspecific fluorescence recording. Signal obtained after such background correction was considered as antigen-specific signal. All sections for a specific receptor antigen were evaluated under identical camera settings so that comparison could be made between groups. Immunofluorescence localization studies were repeated at least three times using tissues from different animals to verify the reproducibility of the data. Representative sections were digitally photographed. Photomicrographs were arranged using Adobe Photoshop (San Jose, CA) image editing software without any further adjustment to maintain the true nature of the findings.

### *Immunoblotting evaluation of ER-α and ER-β protein levels in ovarian cell compartments*

Ovaries of proestrous (D4:0900 h) hamsters were used to verify the specificity of the ER-α antibody, and ovaries of diestrous (D3:0900 h) were used to verify ER-β antibody as well as detect ER proteins in different ovarian cell compartments. After dissecting all large antral follicles and any remnant of regressed corpora lutea, ovaries were treated with collagenase as described by Roy and Greenwald (53) to dissociate most of preantral follicles from interstitial cell compartment. Ovarian digest was filtered through a 350-μm nylon mesh to separate all preantral follicles from nonfollicular compartment. The retentate on the mesh was considered as crude interstitial tissues, which contained a few primary follicles (the number could not be counted). Next, antral follicles were separated into pure granulosa cells and thecal shell devoid of granulosa cells. The RIPA lysates of granulosa, thecal, and interstitial cells were prepared as described (50, 54). Hamster uterine homogenate as negative control for ER-β and positive control for ER-α was also prepared similar to that of granulosa cells. Besides uterine homogenate, recombinant ER-α protein (0.25 μg) and MCF-7 cell lysate (30 μg) were also used as positive controls for ER-α and for both ER, respectively, as suggested by the antibody manufacturers. Equal amount of protein of all lysates were fractionated in 10% polyacrylamide gels, electrotransferred to Optitran membrane and probed with the same ER-α- or ER-β-specific antibody that was used in immunofluorescence study and corresponding second antibody-peroxidase conjugate. To check the specificity of the ER-α immunoblotting, one of the membranes carrying all samples was probed with ER-α antibody preabsorbed with 100-fold excess recombinant human ER-α. Preabsorbed ER-β antibody was also used for probing; however, no recombinant ER-β protein for electrophoresis could be used because of the unavailability. The signal was generated by chemiluminescence WestFemto substrate and recorded on an x-ray film specified for chemiluminescence (Kodak, Rochester, NY). To verify the purity of granulosa, theca, and interstitial cell preparation, supernatants from all lysate were evaluated for aromatase protein, which is exclusively expressed in the granulosa cells.

Next, to determine the overall ER-α and ER-β protein levels in the ovary, RIPA lysates of ovaries of cyclic as well as Hx hormone-treated hamsters were gel fractionated and immunoblotted as described earlier (32, 36) using anti-ER-α or -ER-β antibody. Following chemiluminescence substrate reaction, the exposure time to x-ray film was kept constant for all membranes for comparison. The membrane was washed thoroughly and subjected to β-tubulin immunoblotting for data normalization. All films were digitized in an imaging densitometer (Bio-Rad Laboratories, Inc., Hercules, CA), and data were normalized against β-tubulin signal, which remained constant across samples. Each group had at least three replicates of samples collected from three different animals. The data for Hx and hormone replacement studies were presented relative to Hx-untreated controls for comparison.

### *RT-PCR cloning of hamster ER-α and ER-β cDNA*

We partially cloned hamster ER-α and ER-β cDNA to obtain species-specific nucleic acid probes to study ER mRNA expression in hamster ovarian cells. The primer pairs for ER-α RT-PCR were designed from a conserved region (171–647 bases) of the N terminal A/B domain of rat

(55), mouse (56), and human (21) ER-$\alpha$ cDNA sequences. Similarly, primers for ER-$\beta$ were designed from a conserved region (158–431 bases) of the N-terminal A/B domain of rat (57), mouse (23), goldfish (58), human (59), and bovine (24) ER-$\beta$ cDNA sequences. This was necessary because mouse cDNA did not hybridize with the hamster ER mRNA. The forward and reverse primers for ER-$\alpha$ were: 5′-CGCCGC-CTACGAGTTCAAC-3′ and 5′-CTCTTAAAGAAAGCCTTGCAGCC-3′, respectively. The forward and reverse primers for ER-$\beta$ were: 5′-CTATGCAGAACCTCAAAAGAGTCC-3′ and 5′-TTCGTGGCTGGA-CAGATATAATC-3′, respectively. All primers were synthesized by Genosys Biotechnologies, Inc. (Woodlands, TX). Total RNA from proestrous hamster ovaries was prepared as described previously (60) and reverse transcribed using ER subtype-specific reverse primer to generate ER-$\alpha$ and ER-$\beta$ cDNA, which was denatured at 94 C for 4 min and amplified for 30 cycles in a thermocycler (MJ Research, Inc., Waltham, MA) using the following conditions: 1 min at 94 C, 1 min at 55 C (ER-$\alpha$) or 45 C (ER-$\beta$), 1 min at 72 C, followed by a 10-min extension at 72 C. The final $Mg^{2+}$ concentration was 2.8 mM for ER-$\alpha$ and 3.0 mM for ER-$\beta$. A predicted 474-bp ER-$\alpha$ and a 273-bp ER-$\beta$ cDNA were obtained following PCR amplification. The cDNA was inserted in PCR-II-TOPO vector, transformed in TOPO 10 cells and positive clones were analyzed for the presence of ER cDNA by restriction digestion as well as PCR. Hamster ER cDNA was sequenced in an automated DNA sequencer (UNMC Genetic Sequence Core). Plasmid DNA containing the desired insert was linearized to obtain template for antisense or sense riboprobe synthesis.

### *Northern hybridization detection of hamster ER-$\alpha$ and ER-$\beta$ in the ovary*

Northern hybridization of hamster ER-$\alpha$ and ER-$\beta$ was done essentially as described by Roy (60). Poly[$A^+$] RNA of hamster ovarian, uterine, and leg muscle was prepared using RNAeasy total and Oligotex poly [A+] RNA extraction kits as suggested by the manufacturer (QIAGEN, Valencia, CA). Two and one-half micrograms of each of poly [A+] RNA was fractionated in a denaturing formaldehyde gel, transferred to Nytran membrane (Schleicher & Schuell, Inc.) and UV cross-linked. The membrane was stained briefly with methylene blue to mark the positions of an RNA ladder, hybridized overnight with [$^{32}$P]-antisense or sense cRNA at 68 C in the presence of 50% formamide in Ultrahyb solution (Ambion, Inc., Austin, TX), washed stringently at 68 C, and exposed to x-ray film (Kodak) for 48 h. Approximate size of ER transcripts was calculated from the relative positions of RNA size markers (Invitrogen, Carlsbad, CA).

### *In situ hybridization localization of spatiotemporal distribution of ER-$\alpha$ and ER-$\beta$ mRNA in hamster ovarian cells*

*In situ* hybridization was done as described by Das *et al.* (61) with some modifications. Briefly, frozen sections of ovaries were dried on hot plate at 45 C and fixed in 4% fresh paraformaldehyde in PBS, pH 7.4, on ice for 10 min, followed by three 10-min rinses with ice-cold PBS. After acetylation for 10 min, sections were rinsed in 4× saline sodium citrate (SSC) and dehydrated through ascending grades of ethanol at room temperature. Sections were prehybridized in 50% formamide for 30 min at 37 C, followed by 4-h hybridization in 6× SSC, 10 mM $NaH_2PO_4$, 50 $\mu$g/ml yeast tRNA, 1% dextran sulfate, 50% formamide, 1 mM dithiothreitol, and $2 \times 10^7$ cpm [$^{35}$S]-antisense or sense cRNA at 45 C in a humidified chamber. After thorough rinsing in 4× SSC, nonhybridized probe was removed by RNaseA digestion, followed by rinsing in 1× SSC for 30 min and dehydration in ascending grades of ethanol. Sections were finally coated with NTB2 nuclear track emulsion diluted 1:1 with 0.3 M Na-acetate, dried, exposed in the dark for optimum time, developed in 1:1 dektol (Kodak) in water, stained with hematoxylin and eosin sequence, and mounted in DPX (BDH, Poole, UK). All sections were evaluated under bright- as well as dark-field illumination for silver grain distribution as an index of mRNA expression.

Because ER-$\alpha$ mRNA expression occurred in all cell types and was rather scattered, inverted bright-field photomicrographs had to be presented to differentiate silver grain distribution between follicular and nonfollicular cells. This approach was not necessary for ER-$\beta$ mRNA because major expression of the receptor mRNA was localized in granulosa cells. Finally, *in situ* signal density in granulosa cells of preantral, antral, and atretic follicles and thecal and interstitial cells was quantified using NIH Image version 1.6 image analysis software. There were four or more replicates for each type of follicle and thecal and interstitial cells from each section, and there were two different sections from two animals for each group. Preantral follicles at stages 5 and 6 and antral follicles regardless of the size were used for quantification. No attempt was made to quantify small preantral follicles because we could not identify adequate number with morphological certainty for statistical analysis. The results were expressed as OD per pixel.

Immunolocalization and immunoblotting studies were repeated at least three times, and *in situ* hybridization was repeated twice to ensure reproducibility and verify statistical significance whenever appropriate. Quantitative data were analyzed by two-way ANOVA and Scheffé's F test (StatView, Abacus Concepts, Berkeley, CA) to determine the level of significance ($P < 0.05$).

## Results

For immunofluorescence microphotography, antigen-specific signal was overlaid on nucleus-specific signal to identify nuclear or nonnuclear localization of the ER subtypes. Because ER-specific fluorescence was green and corresponding nuclear signal was red, overlaying resulted in a yellow color. The shade of the yellow color changed to greenish with increasing expression of ER-$\alpha$ protein and finally became green when a very high level of receptor protein was present. Regardless of the blocking solution and mode of blocking, there was a certain degree of autofluorescence, the intensity of which varied depending on the fixative and the type of antibody used. Unfortunately, a same fixative was not useful for both of the antibodies. Further, monoclonal IgG (Fig. 1) appeared to produce less autofluorescence than that of the polyclonal IgG (Fig. 2), but monoclonal ER-$\beta$ antibody suitable for immunofluorescence was not available. However, virtually no nuclear signal was observed when antibodies were preabsorbed with respective antigen, suggesting the specificity of nuclear immunofluorescence. The presence of noticeable background fluorescence was also reported previously (44, 52). Therefore, nonnuclear autofluorescence was subtracted as best as possible without significantly compromising actual nuclear signal. Nevertheless, because of the autofluorescence and very tight organization of ovarian tissue, the specificity of any nonnuclear antibody-specific signal could not be verified; hence, no attempt was made to analyze on any nonnuclear signal.

### *ER-$\alpha$ protein expression in the ovary*

ER-$\alpha$ was present in the nucleus of granulosa, theca, and interstitial cells; however, the intensity of immunoreactivity was more for interstitial cells (Fig. 1). Moderate intensity (reddish yellow) of ER-$\alpha$ immunoreactivity was detectable in granulosa and thecal cells of small preantral follicles and in interstitial cells up to d 3:0900 h (Fig. 1A). Immature granulosa cells of primordial follicles had low, but discernible, ER-$\alpha$ immunoreactivity (Fig. 1A, *inset).* Most notably, a regionalized expression of ER-$\alpha$ in granulosa cells was evident in follicles with incipient antrum (stage 7; Ref. 53) on d 3:0900 h (Fig. 1B). Considerable ER-$\alpha$ immunofluorescence was located in granulosa cells in and around the forming antrum, whereas cells, away from the antrum, had very low signal (Fig. 1B). ER-$\alpha$ immunoreactivity increased markedly (green-

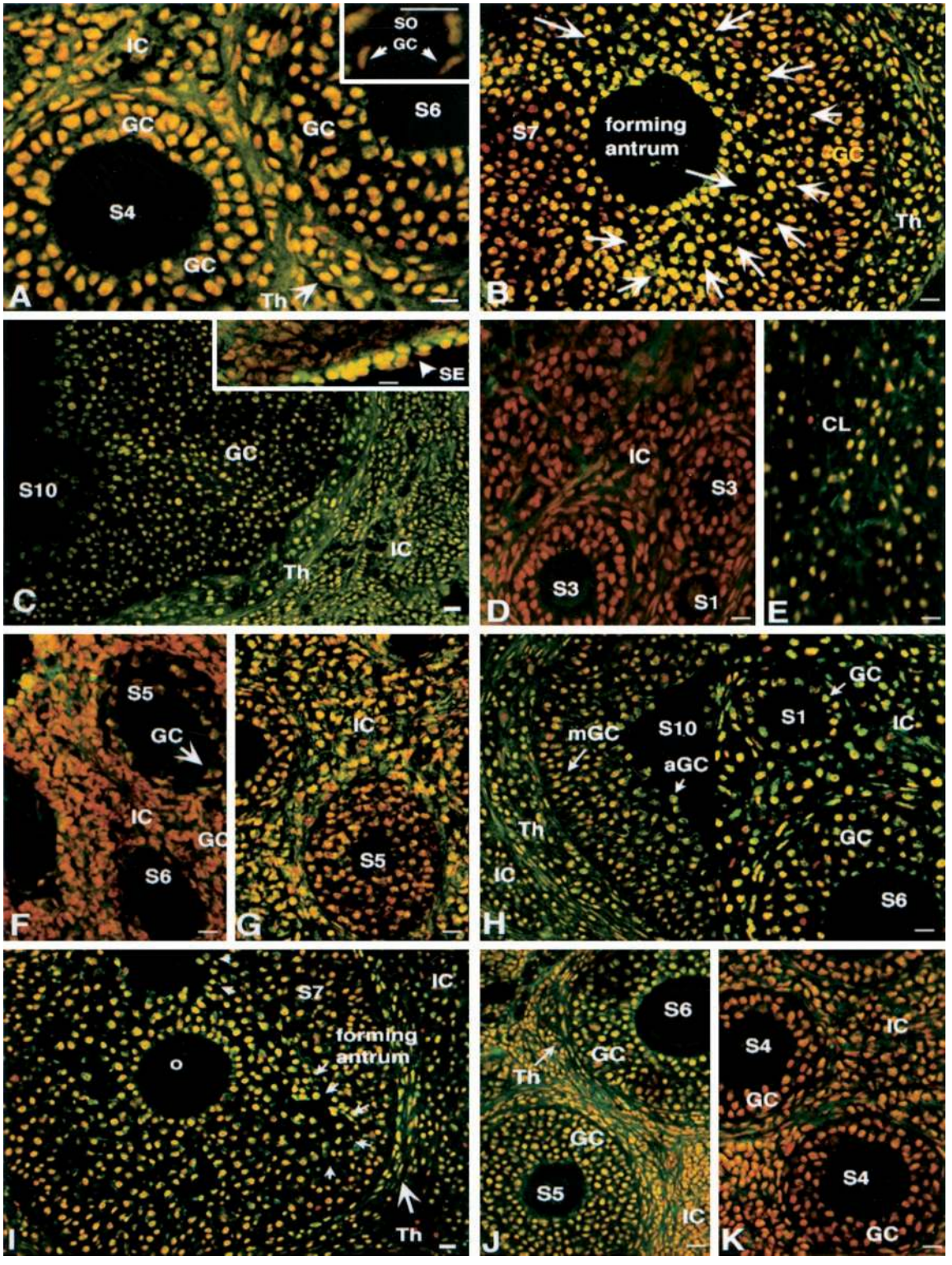



FIG. 1. Photomicrographs showing ER-$\alpha$ immunoreactivity in hamster ovarian cells during the estrous cycle, following hypophysectomy and hormone replacement. A, D 2:0900 h. *Inset* showing ER-$\alpha$ expression in a primordial follicle. B, Note higher regionalized expression of ER-$\alpha$ in granulosa cells in and around the forming antrum *(encircled by arrows),* compared with cells distal to the antrum on d 3:0900 h. C, D 4:0900 h. *Inset* showing ER-$\alpha$ positive surface epithelium. D, D 3:0900 h, antibody preabsorbed with recombinant ER-$\alpha$. E, ER-$\alpha$ positive luteal cells on d 2:0900 h. F, Hx vehicle treated. G, Hx ovary exposed to hCG. H, FSH-exposed Hx ovary. I, Regionalized antrum-specific ER-$\alpha$ expression in follicles developed following FSH treatment *(encircled by arrows).* J, E2-exposed Hx ovary. K, P-exposed Hx ovary. *Bars*, 10 $\mu$m. GC, Granulosa cells; Th, thecal cells; IC, interstitial cells; S0, primordial follicles; S3-S4, preantral follicles with three and four layers of GC, respectively; S6, large preantral follicles with seven to eight layers of GC; S7, follicles with forming (incipient) antrum; S10, large antral follicle; CL, corpus luteum; SE, surface epithelium; mGC and aGC, mural and antral granulosa cells.

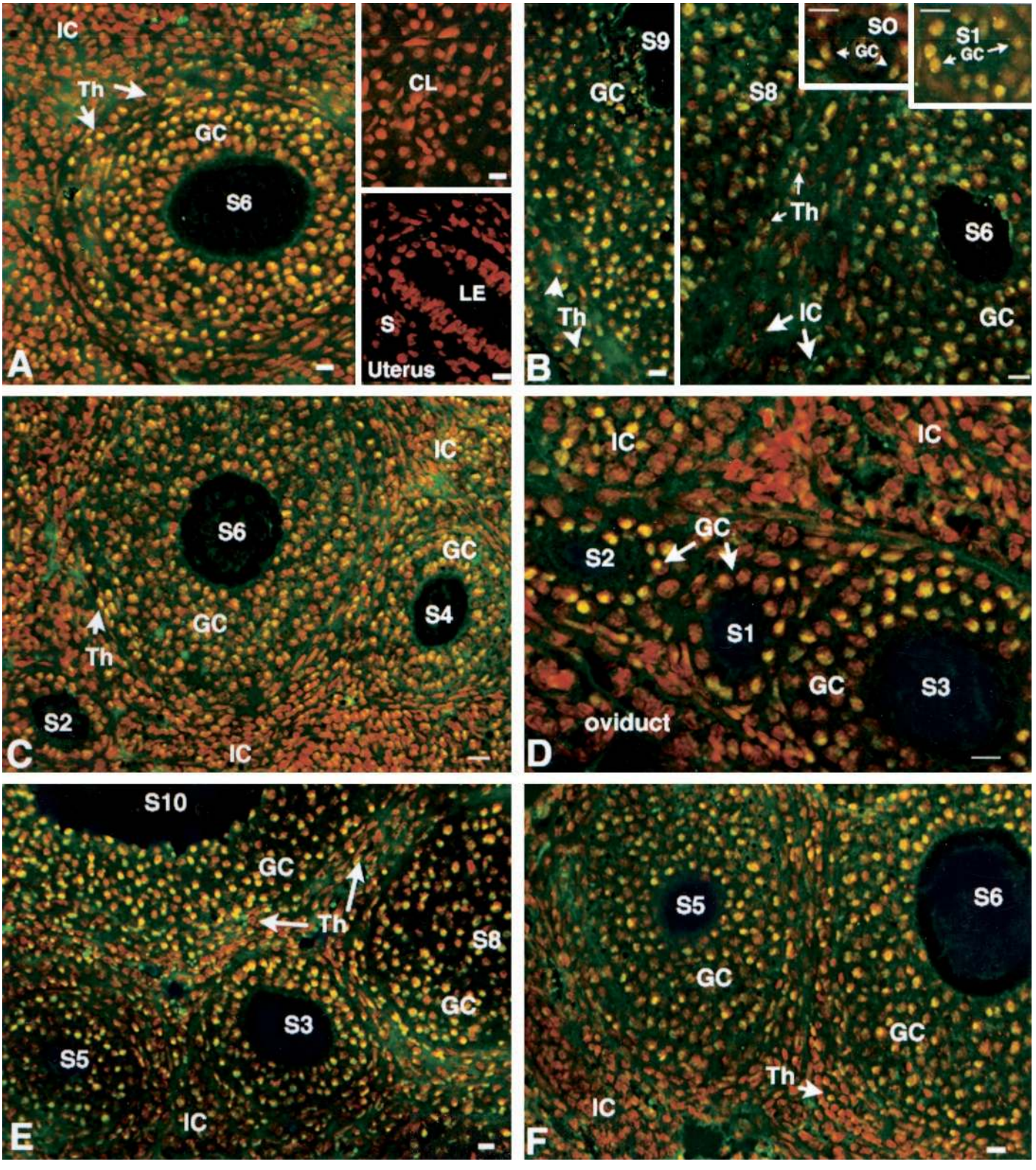



FIG. 2. Photomicrographs showing immunolocalization of ER-$\beta$ protein in hamster ovarian cells during the estrous cycle, following hypophysectomy and hormone replacement. A, D 2:0900 h. *Insets* showing sections of a corpus luteum and uterus. B, D 3:0900 h. *Inset* showing sections of primordial and primary follicles. C, D 4:0900 h. D, Hx vehicle treated. No signal was present in the oviduct. E, FSH-exposed Hx ovary. F, E2-exposed Hx ovary. *Bars*, 10 $\mu$m. GC; Granulosa cells; Th, theca cells; IC, interstitial cells; S0, primordial follicles; S1-S4, preantral follicles with one to four layers of granulosa cells, respectively; S5-S6, preantral follicles with five to six and seven to eight layers of granulosa cells, respectively; S8-S10, antral follicles; LE, luminal epithelium; S, stroma.

ish to green) in granulosa, theca, and interstitial cells on d 4:0900 h whereas moderate immunoreactivity was localized in cells of the surface epithelium (Fig. 1C and *inset).* Further, luteal cells also expressed detectable ER-α immunoreactivity (Fig. 1E). ER-α expression patterns remained almost unchanged following the periovulatory gonadotropin surge (data not shown). ER-α-specific immunoreactivity was virtually absent in a section of d 4:0900 ovary that was incubated with antibody preabsorbed with recombinant ER-α protein (Fig. 1D), indicating the specificity of the immunoreaction. The result was similar for ER-β, when sections of d 3:0900 h ovary was incubated with the antibody preabsorbed with antigen peptide (data not shown).

Hypophysectomy resulted in cessation of follicular growth beyond the preantral stages, which correlated with marked decrease in ER-α immunoreactivity in all cell types (Fig. 1F); however, appreciable increase occurred primarily in interstitial cells in hCG-treated ovaries (Fig. 1G). FSH replacement resulted in a marked increase in ER-α protein expression in granulosa cells of preantral and antral follicles and thecal and interstitial cells (Fig. 1H). Further, antral granulosa cells appeared to have more ER-α immunoreactivity relative to their mural counterpart (Fig. 1H). The regionalized expression of ER-α in granulosa cells in and around the forming antrum was also evident following FSH-induced follicular development; however, the intensity of immunosignal was appreciable more than that of Fig. 1B (Fig. 1I). No change in FSH-induced ER-α expression was noted when hCG was administered simultaneously (data not shown). Similar to FSH, E2 significantly stimulated ER-α expression in granulosa, theca, and interstitial cell compartments (Fig. 1J). Progesterone (P) alone did not influence ER-α expression appreciably (data not shown); however, it significantly attenuated E2-induced increase in ER-α expression in granulosa and interstitial and (Fig. 1K).

### *ER-β protein expression in the ovary*

In contrast to ER-α, ER-β immunosignal was primarily restricted to granulosa cells of all follicles on d 1:0900 h; however, low level of immunoreactivity was present in a few interstitial and thecal cells (Fig. 2A). However, no signal was detectable in cells of the corpus luteum or uterus (Fig. 2A). Significant increase in ER-β immunoreactivity occurred primarily in granulosa cells of preantral and antral follicles by d 3:0900 h, but some thecal and interstitial cells also showed intense immunoreactivity (Fig. 2B). Further, considerable ER-β expression was evident in granulosa cells of primordial and primary follicles as well (Fig. 2B, *inset).* Unlike ER-α, ER-β expression declined considerably on d 4:0900 h in granulosa cells of preantral follicles and in interstitial cells (Fig. 2C) and remained low following the periovulatory gonadotropin surge (data not shown).

Following Hx, low levels of ER-β immunoreactivity remained in granulosa cells of preantral follicles, whereas signal intensity in interstitial cells was barely detectable (Fig. 2D). FSH-induced follicular development coincided with significant increase in ER-β immunoreactivity in granulosa cells of preantral and antral follicles (Fig. 2E). Many cells of the interstitium and theca also showed distinct immunosignal (Fig. 2E). Similar to FSH, estrogen replacement resulted in an appreciable increase in ER-β immunoreactivity, primarily in granulosa cells (Fig. 2F). Neither hCG nor P alone stimulated ER-β expression in granulosa or interstitial cells (data not shown), but hCG attenuated FSH-induced and P attenuated E2-induced ER-β immunoreactivity to the level detected in ovaries of untreated, Hx hamsters (Fig. 2D).

### *ER-α and ER-β protein levels in ovarian cells*

Immunoblotting studies revealed an approximately 54-kDa ER-β and an approximately 68-kDa ER-α bands, respectively, in granulosa, theca, and interstitial cell lysates (Fig. 3, A and C). Although the interstitial cell preparation might have a few small preantral follicle contaminations, the presence of almost equal level of ER-β in interstitial cell preparation, compared with pure granulosa cell preparation, clearly indicates the definite presence of ER-β in interstitial cells (Fig. 3A). The purity of thecal cell preparation was evident from the absence of aromatase protein (Fig. 3B), which was expressed solely in granulosa cells (62, 63) of large preantral (stages 5 and 6; Ref. 53) and antral follicles. The absence of ER-β signal (Fig. 3A, *lane 4),* and the presence of ER-α signal (Fig. 3C, *lane 4)* in the uterine lysate indicated the specificity of the antibodies. Human ER-β appeared to be slightly larger than that of hamster because the antibody detected an approximately 65-kDa band in MCF-7 cell lysate (Fig. 3A, *lane 5)* as indicated by the antibody manufacturer. On the other hand, the size of human and hamster ER-α appeared to be very similar as evident from the signal emanating from the MCF-7 cell lysate and recombinant ER-α protein, respectively (Fig. 3C, *lanes 5* and *6).* ER-α-specific signal for all tissue and cell lysates disappeared completely, and significant reduction was noted for the recombinant ER-α when the membrane was probed with the antibody preabsorbed with recombinant ER-α (Fig. 3D). A similar finding was observed when the membrane was probed with ER-β antibody preabsorbed with the antigen peptide (data not shown).

Overall ER-α protein levels in the hamster ovary were low up to d 2:0900 h (Fig. 4A) when FSH levels were on the rise (64). ER-α levels started to increase by d 3:0900 and increased significantly (~6-fold, compared with d 1:0900 h level, $P < 0.05$) by d 4:0900 h (Fig. 4A) coinciding with low serum FSH and high serum E2 levels (62, 64). Receptor levels remained unchanged following the preovulatory gonadotropin surge. Ovarian ER-β protein levels appeared to be at least two times more than that of ER-α. In contrast to ER-α, ovarian ER-β protein levels increased considerably by d 2:0900 h and reached a significantly high ($P < 0.05$) level on d 3:0900 h (Fig. 4A) coinciding with serum FSH levels (64). ER-β expression decreased significantly ($P < 0.05$) by d 4:0900 h when serum estrogen levels were high (50) but remained higher than d 1:0900 h value (Fig. 4A). Ovarian ER-α and ER-β levels were low (OD: 0.48 ± 0.03 and 0.39 ± 0.07, respectively) in Hx hamsters. Whereas FSH treatment significantly increased ($P < 0.05$) the levels of both receptor subtypes, the stimulatory effect of hCG was limited to ER-α (Fig. 4B). However, concurrent administration of hCG with FSH resulted in sig-

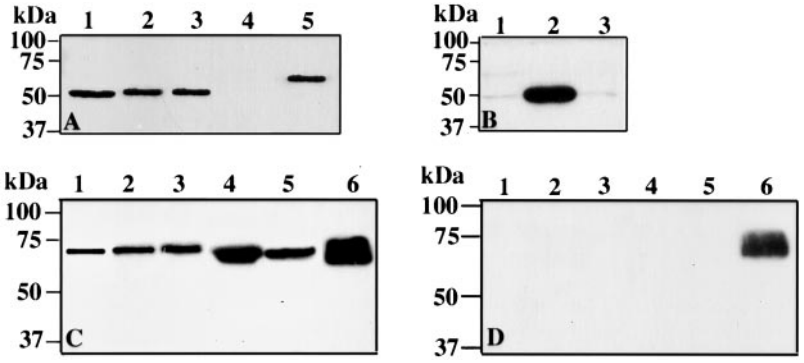



FIG. 3. Western immunoblotting detection of ER-$\beta$ (A), aromatase enzyme (B), and ER-$\alpha$ (C-D) in lysate of interstitial cells *(lane 1),* granulosa cells *(lane 2),* thecal cells *(lane 3),* uterus *(lane 4),* MCF-7 cells *(lane 5),* and recombinant human ER-$\alpha$ protein *(lane 6).* Granulosa, thecal, and interstitial cells were isolated on d 3:0900 h for ER-$\beta$ and on d 4:0900 for ER-$\alpha$. D, Membrane was probed with ER-$\alpha$ antibody preabsorbed to antigen.

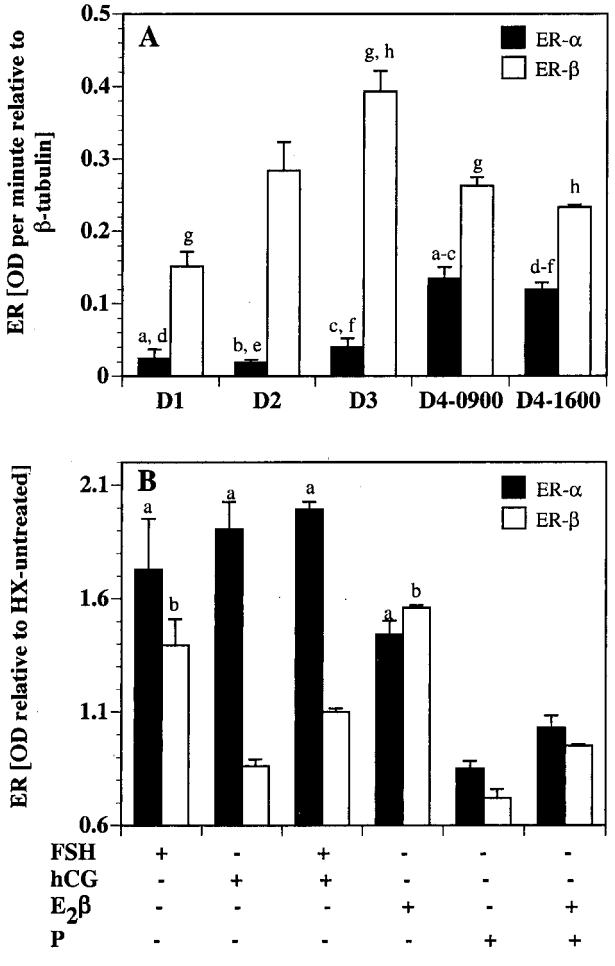



FIG. 4. Immunoblot analysis of ER-$\alpha$ and ER-$\beta$ protein in the hamster ovary throughout the estrous cycle (A) and following hypophysectomy and hormone replacement (B). Note differential expression patterns of ER subtypes during the estrous cycle and following the periovulatory gonadotropin surge. Values with *same letters* were significantly ($P < 0.05$) different from each other.

nificant ($P < 0.05$) attenuation of ER-$\beta$ level, but no such inhibition was evident for ER-$\alpha$ (Fig. 4B).

E2 exposure for 24 h led to significant ($P < 0.05$) increases in levels of both receptor subtypes (Fig. 4B). Although P alone did not affect ovarian ER-$\alpha$ or ER-$\beta$ (Fig. 4B) protein expression, significant attenuation of E2-induced ER-$\alpha$ and ER-$\beta$ protein expression was evident when P was administered to E2-primed Hx hamsters (Fig. 4B).

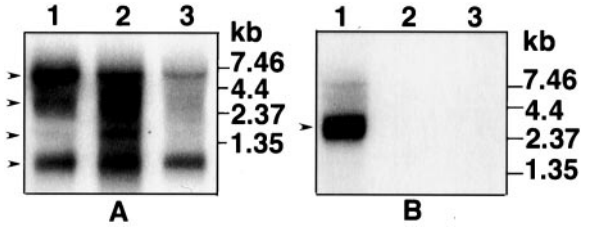
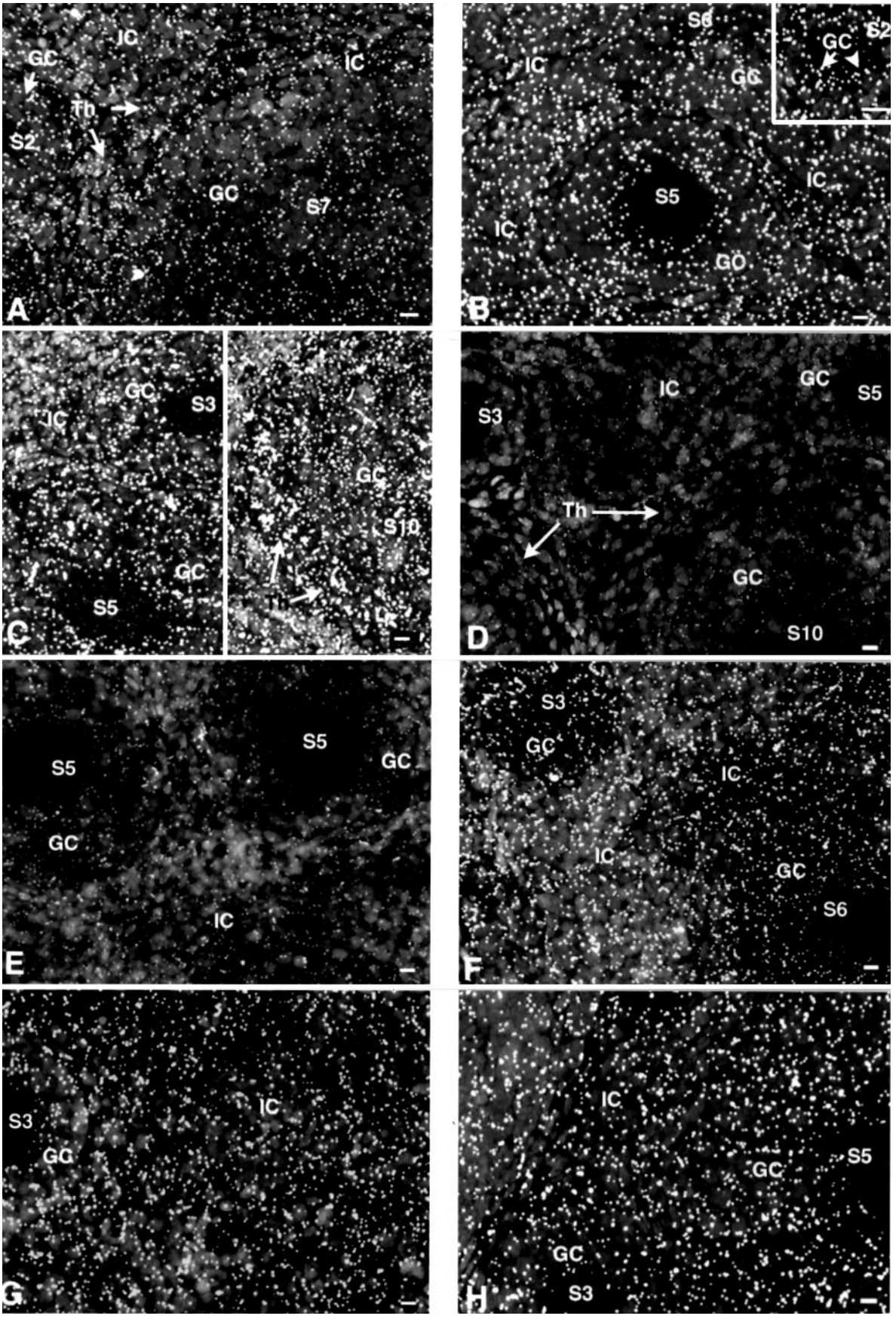



FIG. 5. Northern hybridization detection of ER-$\alpha$ (A) and ER-$\beta$ (B) messenger RNA in hamster ovarian Poly [$A^+$] *(lane 1),* uterine *(lane 2),* and muscle *(lane 3)* RNA.

### *ER-α and ER-β mRNA levels in the hamster ovary and the effects of hormones*

Northern hybridization revealed four ER-$\alpha$ transcripts of approximately 7.0, 3.6, 1.9, and less than 1.35 kb (Fig. 5A) in ovarian poly [$A^+$] RNA. ER-$\alpha$ transcripts of 7.0, 3.6, and less than 1.35 kb were expressed in the ovaries as well as in the uterus; however, 1.9-kb transcript represented a minor band in the ovary relative to the uterus. On the other hand, a major more than 1.35 kb and a minor 7.0 bands were visible for the muscle. In contrast to ER-$\alpha$, a minor transcript of 7.4 kb and a major transcript of 2.95 kb for ER-$\beta$ were identified in ovarian poly [$A^+$] RNA preparation (Fig. 5B). The ER-$\beta$ cRNA did not detect any transcript in either uterus or muscle, indicating the specificity of the ER nucleic acid probes. Further, no signal was obtained when membrane was probed with [$^{32}$P]-labeled sense probe (data not shown), indicating the specificity of the hybridization. Sequence comparison revealed that hamster ER-$\alpha$ cDNA was 93%, 89%, and 84%, similar to the corresponding region of the mouse, rat, and human ER-$\alpha$ cDNA sequences, respectively. Further, our PCR-derived hamster ER-$\alpha$ sequence was 99.8%, similar to that reported by Bhat *et al.* (65). Hamster ER-$\beta$ cDNA was 91%, 90%, 88%, 88%, 77%, and 75%, similar to the corresponding region of the rat, mouse, human, bovine, and goldfish ER-$\beta$ cDNA sequences, respectively.

*In situ* hybridization studies revealed that both ER-$\alpha$ (Fig. 6, A–C) and ER-$\beta$ (Fig. 7, A–G) mRNA was expressed in granulosa cells of preantral and follicles. Overall, in contrast to ER-$\beta$ hybridization signal (Fig. 7), ER-$\alpha$ signal was scattered all over the ovary, which was not surprising, considering its site of expression as reported for other species (3); hence, differentiating tissue boundaries was challenging. Therefore, clear-cut pictures similar to ER-$\beta$ (Fig. 7) could not be obtained. Nevertheless, discrete ER-$\alpha$



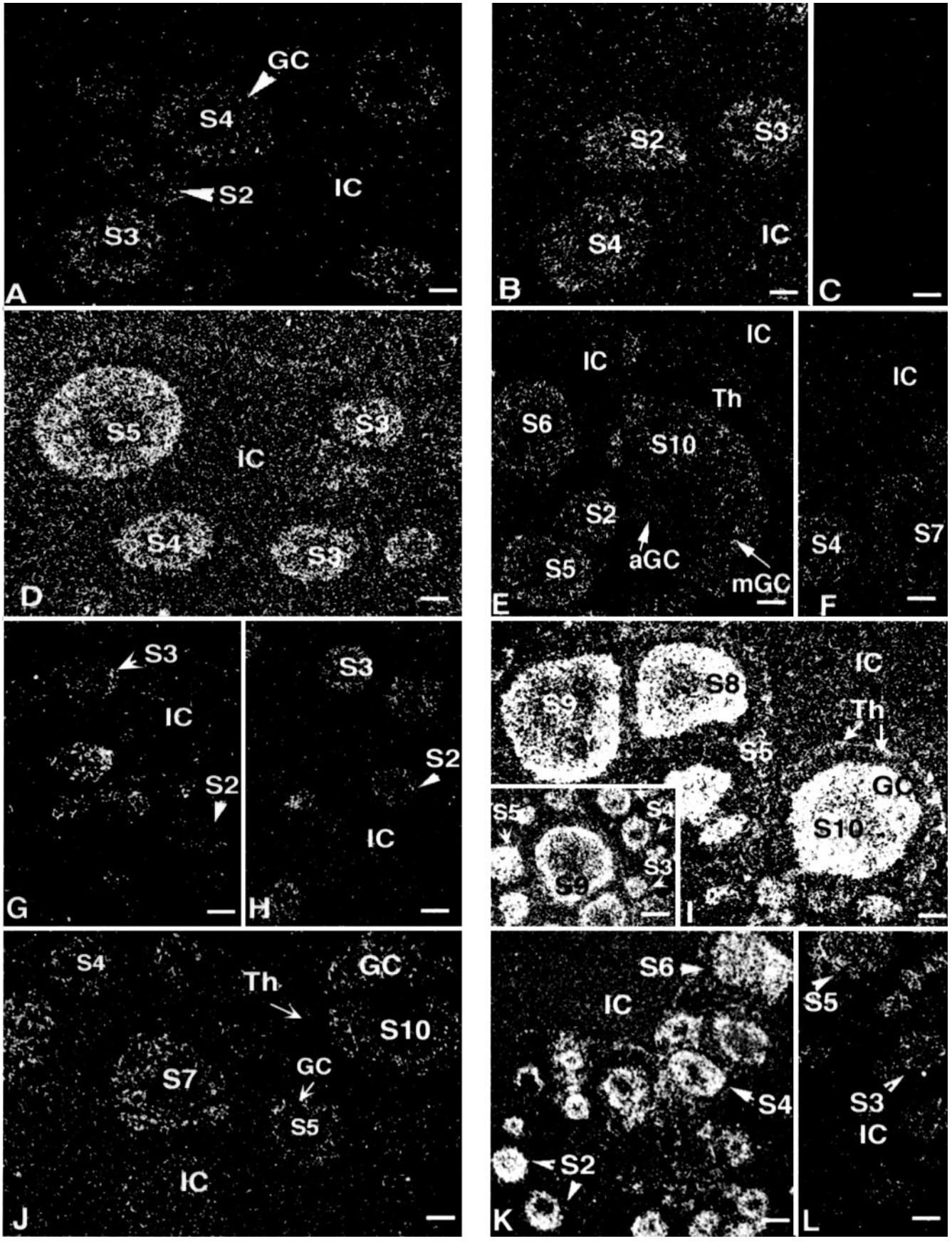
mRNA was present in granulosa cells, but the expression was noticeably higher in interstitial and thecal cells (Figs. 6, A–C, and 8A), thus corroborating with the immunofluorescence data. The level of mRNA expression was low on d 2:0900 h (Figs. 6A and 8A) but increased noticeably in all cell types on d 3:0900 h, especially in interstitial and thecal cells (Figs. 6B and 8A). Granulosa cells of small preantral follicles (S2, Fig. 6B, *inset)* showed distinct expression of ER-$\alpha$ transcript. A marked increase in ER-$\alpha$ mRNA expression occurred in interstitial, theca, and granulosa cells on d 4:0900 h (Figs. 6C and 8A). No further increase was apparent following the gonadotropin surge at d 4:1600 h (Fig. 8A). Sections of d 4:0900 h ovary exposed to [$^{32}$P]-sense ER-$\alpha$ cRNA showed no specific hybridization signal (Fig. 6D), indicating the specificity of the hybridization localization. A low amount of silver grains was associated with granulosa and interstitial cells in ovaries of Hx hamsters (Figs. 6E and 8B). Marked increases in ER-$\alpha$ mRNA expression in preantral and antral granulosa cells and in interstitial cells was noted following FSH treatment (Figs. 6F and 8B). On the other hand, hCG or E2 induced ER-$\alpha$ mRNA expression in preantral follicles and interstitial cells (Figs. 6, G–H, and 8). ER-$\alpha$ mRNA expression in antral granulosa cells increased significantly ($P < 0.05$) but decreased markedly in interstitial cells following a combined treatment of FSH and hCG (Fig. 8B).

Low levels of ER-$\beta$ expression were evident in granulosa and nongranulosa cells on d 1:0900 h (Figs. 7A and 8C). ER-$\beta$ mRNA expression increased considerably on d 2:0900 h primarily in granulosa cells of preantral (Figs. 7B and 8C) and antral (Fig. 8C) follicles. A marked increase in ER-$\beta$ mRNA expression in preantral (Figs. 7D and 8C) and antral (Fig. 8C) granulosa cells occurred on d 3:0900 h, followed by a noticeable decline on d 4:0900 h (Figs. 7E and 8C), which continued through d 4:1600 h (Figs. 7F and 8C). The expression of ER-$\beta$ mRNA in interstitial cells also increased by d 3:0900 h and decreased thereafter (Fig. 8C). No expression of ER-$\beta$ mRNA could be detected when sections were hybridized with sense cRNA (Fig. 7C). In Hx hamster ovaries, ER-$\beta$ mRNA expression was low in granulosa cells of preantral follicles and in interstitial cells (Figs. 7G and 8D). Administration of hCG failed to significantly influence ER-$\beta$ transcript level either in granulosa or interstitial cells (Figs. 7H and 8D). On the contrary, FSH replacement significantly stimulated ER-$\beta$ mRNA expression in granulosa cells of follicles in all categories (Figs. 7I, *inset,* and 8D). Marked increase in ER-$\beta$ mRNA expression was also evident for interstitial cells (Figs. 7I and 8D, compared with Hx-control (Figs. 7G and 8D) or hCG-treated groups (Figs. 7H and 8D). Interestingly, concurrent injection of hCG dramatically reduced FSH-stimulated ER-$\beta$ mRNA expression (Figs. 7J and 8D). Similar to FSH effect, significant induction of ER-$\beta$ mRNA expression occurred primarily in granulosa cells of preantral follicles at all categories following E2 administration (Figs. 7K and 8D). Receptor mRNA expression was also visible in interstitial cells adjacent to follicles (Fig. 7K), but the increase was moderate (Fig. 8D). Whereas the effect of P alone on ER-$\beta$ mRNA expression was no different from that of hCG (Figs. 7H and 8D), administration of P in E2-primed hamsters caused significant attenuation of the stimulatory effect of E2 on receptor gene expression (Figs. 7L and 8D).

## Discussion

The results of these studies establish the spatiotemporal pattern of ER-$\alpha$ and ER-$\beta$ protein and mRNA expression in hamster ovarian cell types and provide strong evidence for the differential regulation of receptor subtype expression by gonadotropins and ovarian steroids. Further, immunofluorescence findings correlate well with quantitative immunoblotting and *in situ* hybridization data. The results of the present studies provide clear evidence for the first time that both ER-$\alpha$ and ER-$\beta$ are expressed in granulosa cells of primordial follicle stage onward; however, the level of translation increases noticeably when flattened granulosa cells differentiate to form cuboidal granulosa cells of primary follicles. ER-$\alpha$ protein and mRNA cannot be detected in mouse granulosa cells (3, 66), but the presence of ER-$\alpha$ immunoreactivity has been demonstrated in granulosa cells of rat (34, 36), monkey (40, 41), and human antral follicles (43). Further, Sharma *et al.* (44) have demonstrated that cultured rat granulosa cells express ER-$\alpha$ *in vitro,* and the expression is stimulated by Forskolin. On the other hand, Fitzpatrick *et al.* (34) and Sar and Welsh (30) have failed to detect any ER-$\alpha$ protein in granulosa cells of rat ovarian sections by immunohistochemistry. Therefore, the use of methodologies and tools seems to play an important role in the outcome of cell- or tissue-specific localization of ER subtypes. The use of multiple different integrated approaches in the present study verifies the specificity of ER subtype localization in hamster ovarian cells, and the data become consistent with that reported for the primate and human ovarian cells (3). Nevertheless, these lines of evidence not only highlight diversity in ER-$\alpha$ expression among species but also suggest that the ER-$\alpha$ gene is expressed in granulosa cells when differentiation-inducing stimulus is applied. Similarly, the presence of discrete ER-$\beta$ immunoreactivity in thecal and interstitial cells of monkey ovary has been demonstrated (67). Although granulosa cells are the primary sites of ER-$\beta$ expression, coexpression of ER-$\beta$ with relatively higher expression of ER-$\alpha$ in theca and interstitial cells suggests that both subtypes must be necessary for normal ovarian functions. Deletion of ER-$\alpha$ in mice results in ovulation failure (46), which has been ascribed to tonic high levels of LH (47). However, $\alpha$ERKO mice ovulate fewer eggs, compared with the wild-type, in response to superovulatory stimulus, and $\beta$ERKO

FIG. 6. Photomicrographs of *in situ* hybridization localization of ER-$\alpha$ mRNA expression in the hamster ovary during the estrous cycle, following hypophysectomy and hormone replacement. A, D 2:0900 h. B, D 3:0900 h. *Inset* showing a stage 2 preantral follicle. C, D 4:0900 h. D, D 4:0900 h; ovary section was exposed to sense ER-$\alpha$ cRNA. E, Hx vehicle treated. F, FSH-exposed Hx ovary. G, Hx ovary exposed to hCG. H, E2-exposed Hx ovary. *Bars*, 10 $\mu$m. GC, Granulosa cells; Th, theca cells; IC, interstitial cells; S2-S3, preantral follicles with two to three layers of granulosa cells, respectively; S5, preantral follicles with five to six layers of granulosa cells; S6, large preantral follicles with seven to eight layers of granulosa cells; S7, follicle with forming antral cavity.



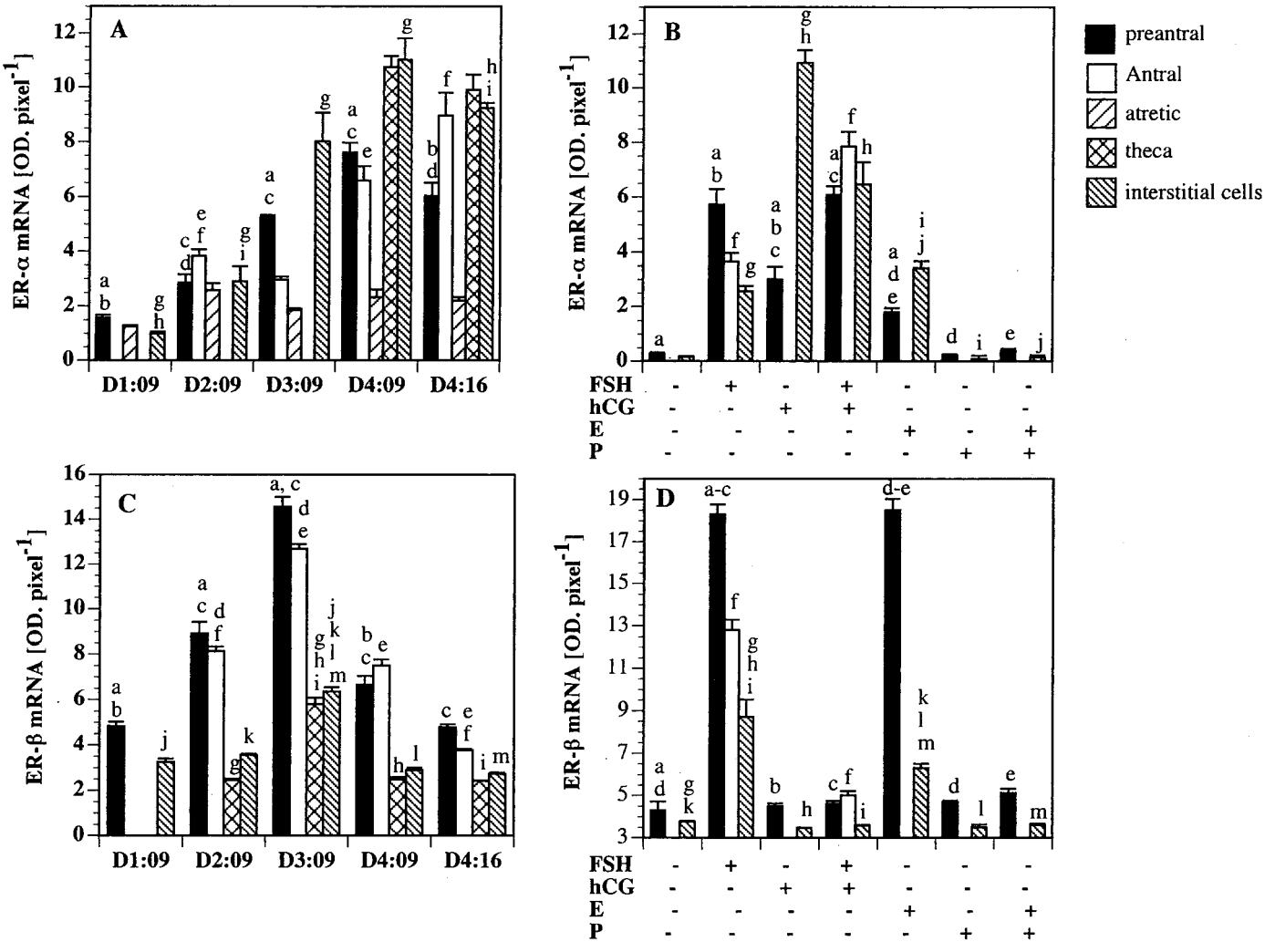



FIG. 8. Quantitative analysis of *in situ* hybridization signal presented in Figs. 6 and 7. ER-α and ER-β mRNA expression in the ovary during the estrous cycle (A and C) and following hypophysectomy and hormone replacement (B and D). Values with *same letters* were significantly ($P < 0.05$) different from each other in respective graph.

mice response even poorer than αERKO mice (46, 48). Dupont *et al.* (49) have not only confirmed the poor ovulatory response of αERKO mice but also demonstrated that antral follicles in both βERKO and αβERKO mice are significantly deficient of granulosa cells. Further, αβERKO mice fail to ovulate in response to exogenous gonadotropins (49). In contrast, mice null with the CYP19 gene have many small antral follicles with adequate granulosa cells but without apparent hemorrhage or cyst formation and no ovulation despite the fact that they also experience higher LH level (68). All these lines of evidence clearly indicate that estrogen via ER may play an important role in granulosa cell maturation, which is critical for successful folliculogenesis beyond the antral stage.

ER-β transcripts of 5–6 kb, 2.5 kb, 1.8 kb, and 0.9 kb have been detected in the rat (22). We have detected one major and a minor ER-β transcript in the hamster ovary, but their sizes correspond well with that of the rat. However, in contrast to a major 6.5-kb ER-α transcript that has been detected in the mouse uterus (56) and rat ovary (22), we have detected three major and a minor ER-α transcript in the hamster ovary poly [$A^+$] RNA; however, the largest size of 7.07 kb of hamster ER-α corresponds well with that of mouse. Interestingly, only one band of approximately 68-kDa ER-α protein has

FIG. 7. Dark-field photomicrographs of *in situ* hybridization localization of ER-β mRNA expression in the hamster ovary during the estrous cycle, following Hx hormone replacement. A, D 1:0900 h. B, D 2:0900 h. C, Section of d 3:0900 h ovary incubated with sense ER-β cRNA. D, D 3:0900 h. E, D 4:0900 h. F, D 4:1600 h. G, Hx vehicle treated. H, Hx ovary exposed to hCG. I, FSH-treated Hx ovary. *Inset* showing ER-β mRNA expression in small preantral follicles developed under FSH influence. J, FSH+hCG-treated Hx ovary. K, E2-treated Hx ovary. L, E2+P-treated Hx ovary. *Bars*, 20 μm. GC, Granulosa cells; Th, theca cells; IC, interstitial cells; S2-S4, preantral follicles with one to four layers of granulosa cells, respectively; S5, preantral follicles with five to six layers of granulosa cells; S6, large preantral follicles with seven to eight layers of granulosa cells; S7, follicle with forming antral cavity; S10, antral follicle.

been detected in hamster ovarian or uterine homogenate, suggesting the possible existence of alternative spliced variants of ER-$\alpha$ transcripts in the hamster reproductive organs. The existence of ER-$\alpha$ spliced variants has been reported in the rat brain (69) and human tissue (70). The presence of all four transcripts in uterine RNA preparation confirms the validity of the data. The use of species-specific nucleic acid probes overrules the possibility of incompatibility between the probe and target mRNA, and the presence of ER-$\alpha$ and absence of ER-$\beta$ mRNA in the uterus validate the specificity of the nucleic acid probes.

The complete overlapping of immunofluorescence and immunoblotting data not only verifies the specificity of the temporal patterns of ER-$\alpha$ and ER-$\beta$ protein expression during the estrous cycle and following hormone replacement but also provides a clear evidence for the differential regulation of receptor subtype expression. Differential expression of ER-$\alpha$ and ER-$\beta$ protein in the rat ovary (31, 34) and FSH/cAMP regulation of ER expression in rat granulosa cells in culture have been documented (44). Although ER subtype expression during the monkey menstrual cycle has been reported (40), information about critical cycle-related changes in ovarian ER expression is not apparent. The results of the present studies in conjunction with the pattern of gonadotropin and steroid hormone changes in intact (50, 64) and hypophysectomized (50) hamsters suggest that gonadotropin and steroid hormone exert differential regulatory influence on ovarian ER subtype expression.

The apparent increase in ER-$\alpha$ but a decreases in ER-$\beta$ expression with the advent of LH surge and following hCG administration correlate well that reported previously (34, 44). Sharma *et al.* (44) have shown that although Forskolin stimulates ER-$\alpha$ and ER-$\beta$ protein expression in unprimed or estrogen-primed rat granulosa cells in culture by 48 h, estrogen has a negative effect. However, Forskolin fails to induce ER-$\beta$ expression in granulosa cells exposed to hCG. Fitzpatrick *et al.* (34) have also documented that ER-$\beta$ expression decreases markedly following hCG administration. The unique temporal expression patterns of ER-$\alpha$ and ER-$\beta$ during the cycle with reference to serum levels of E2 and P (50) suggest that ovarian steroids have a dominant regulatory role on ER expression relative to pituitary gonadotropins. In the hamster, serum P levels start to rise from d 1 to reach a peak on d 2:0900 h, followed by a sharp decline on d 3:0900 h (50). On the contrary, serum E levels fall from d 1 through d 2, followed by a steady increase through d 4:0900 h (50). The results of the present studies suggest that at least part of the FSH-induced ER expression may be mediated by E2; however, P negatively influences ovarian ER expression. This inhibitory effect of P on ER expression may account for lower levels of both ER-$\alpha$ and ER-$\beta$ protein on d 1:0900 h, when plasma progesterone levels increase significantly (50). In light of the inhibitory effect of P on ER mRNA and protein expression, and the unique differential expression patterns of ER-$\alpha$ and ER-$\beta$ proteins and mRNA during the estrous cycle, it may be conjectured that ER-$\alpha$ gene is more sensitive to the inhibitory influence of P because a noticeable increase in ER-$\alpha$ protein or mRNA does not occur until d 4:0900 h when plasma P levels drop to low baseline (50).

Conversely, the ER-$\alpha$ gene may be less sensitive to the stimulatory effect of E2, hence, does not initiate transcription until E2 levels reach very high levels as it happens on d 4:0900 h (50). On the other hand, the ER-$\beta$ gene is more sensitive to the stimulatory effect of E2; hence, P suppression is reversed as soon as follicular E2 production resumes along with the recruitment of antral follicles by d 2 of the cycle. Estrogen stimulation of the data of gonadotropin replacement studies further emphasizes that part of the FSH stimulation of ER-$\alpha$ and ER-$\beta$ protein, and mRNA expression in interstitial cells must be mediated by E2 because FSH receptors are absent in these cells. A similar effect of FSH on hamster interstitial cell TGF-$\beta$ receptor type II protein expression has been documented (50).

Although both ER-$\alpha$ and ER-$\beta$ apparently have the same affinity for E2 (25), considering the differential spatiotemporal expression patterns of these two receptor subtypes in ovarian follicular and nonfollicular cells, different estrogen regulation of cell function is likely. Different effects of ER-$\alpha$ and ER-$\beta$ on the female reproductive system have been demonstrated by selective removal of either gene (49, 66). The results of our studies indicate that although both receptor subtypes are present in ovarian cells, the levels of expression differ significantly among cell types and within cells during the estrous cycle. For example, granulosa cells of preantral follicles express more ER-$\beta$ than ER-$\alpha$; however, increased expression of ER-$\alpha$ occurs as follicles develop through stages. Because hamster preantral follicles cannot *de novo* synthesize E2 until they acquire five to six layers of granulosa cells encircled by developing theca (62), enhanced estrogen action on follicles via coexpression of ER-$\alpha$ in granulosa cells may be necessary to ensure granulosa cells differentiation related to the formation of antral follicles and follicle selection. This is evident from the regionalized increased expression of ER-$\alpha$, but not ER-$\beta$, protein during antrum formation, and the selective presence of ER-$\alpha$ and complete absence of ER-$\beta$ in the corpus luteum, part of which is comprised of differentiated granulosa cells. ER-$\beta$ expression decreases in luteinized granulosa cells and cannot be induced by Forskolin (44). Estrogen has been shown to stimulate ER-$\alpha$ expression in rat granulosa cells in culture followed by a decrease by 48 h (44). Our data on ER protein and mRNA expression in granulosa cells following 24 h of estrogen exposure support this finding and suggest that a gradual increase in estrogen exposure in tandem with follicular growth may be critical for proper maturation of granulosa cells *in vivo*. Our data also indicate that estrogen also regulates its own receptor level in interstitial cells, hence their functionality.

The importance of ER-$\alpha$ in mouse interstitial cell development has been documented (49). Hall and McDonnell (71) have elegantly demonstrated that the activation of expressed ER-$\beta$ in HepG2, HeLa, or 293 cells by subsaturating levels of E2 results in transdominant inhibition of expressed ER-$\alpha$ transcriptional activity and decrease in overall cellular sensitivity to E2. Further, ER-$\alpha$ and ER-$\beta$ can form heterodimers within target cells (71). ER-mediated gene transcription involves the binding of ligand-bound receptor homodimer to the classical estrogen response element (ERE); however, ER also mediates gene transcription from an AP-1 enhancer element that depends on the binding of ligand as well as AP-1 transactivation factors, such as Fos and Jun (72–73). Paech *et*

*al.* (74) have shown that although binding of E2-ER-$\alpha$ complex to either ERE or AP-1 site stimulates transcription, binding of E2-ER-$\beta$-complex to the ERE stimulates but to AP-1 sites inhibits transcription. Although the present results do not provide any direct evidence to suggest whether ER-$\beta$ counteracts ER-$\alpha$ effect in hamster follicular cells, the preferential inhibition of FSH induction of ER-$\beta$ mRNA and protein expression by hCG, and stimulation of ER-$\alpha$ mRNA and protein by both FSH and hCG coincide with the development of antral follicles in Hx hamsters (51), suggesting therefore that inhibition of ER-$\beta$-mediated effect may be essential for E2 to positively influence follicular cells during development. Deletion of ER-$\beta$ gene does not impair the formation of antral follicles in mice (48, 49).

Taken together, all these lines of evidence suggest strongly that the relative expression level and different modes of signaling of the two ER subtypes in follicular cells during folliculogenesis may form a key modulator of cellular response to E2. This contention is particularly relevant to the development of small preantral follicles, in which relatively higher levels of ER-$\beta$ and low levels of E2 (trickling from large preantral and antral follicles) during the beginning of the estrous cycle may prevent E2-induced premature differentiation of follicular cells and allowing E2 support of cell proliferation. Higher expression of ER-$\alpha$ in granulosa cells during the second half of the estrous cycle may override ER-$\beta$ suppression and allow cells to complete postmitotic events and acquire more differentiation functions, such as steroidogenesis, as follicles develop through higher classes. On the other hand, higher ER-$\alpha$ and low ER-$\beta$ expression in interstitial cells may allow E2-induced interstitial cell differentiation, which is necessary for steroidogenesis and supply of thecal cells for folliculogenesis.

In summary, the results of this investigation have demonstrated unique and differential expression patterns of ER subtype protein and mRNA in hamster ovarian cells under endogenous hormonal milieu. The studies of selective hormone replacement in Hx hamsters have provided clear evidence that estrogen up-regulates its receptor expression in the ovary; however, cellular response to E2 is critically modulated by progesterone.

## Acknowledgments

Received November 6, 2001. Accepted February 15, 2002.

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This work was supported by Grant HD-28165 from the National Institute of Child Health and Human Development, NIH.

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