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3β-hydroxy-5-ene steroid dehydrogenase gene expression regulation in porcine granulosa cells. I: FSH- and LH-mediated transcriptional activation

P. Jorge Chedrese, Gheorghe T. Braileanu & Rebecca Salmon

Reproductive Biology Research Unit, Department of Obstetrics and Gynecology, School of Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada

In this report we examined the effect of FSH and LH, on the steady state levels of 3β-5-hydroxy-5-ene steroid dehydrogenase (3β-HSD) mRNA and on the 3β-HSD-gene transcriptional activation in porcine cultured granulosa cells. Exposure of granulosa cells to 100 ng/ml FSH or LH for 8 h, elevated to 3.0 and 2.5-fold respectively the levels of 3β-HSD mRNA measured by Northern blot analyses. The withdrawal of FSH and LH induced a rapid decay of the 3β-HSD levels, reaching the control values after 2 h. Re-addition of FSH and LH after 4 h withdrawal elevated the levels of 3β-HSD mRNA to 4.8 and 5.3-fold respectively. Addition of actinomycin D, to granulosa cells previously treated with FSH or LH, induced a rapid decay in the levels of 3β-HSD mRNA, reaching the control values after 2 h, with an estimated half life 1.3 and 1.2 h respectively. FSH and LH stimulated the 3β-HSD-gene transcription, measured by nuclear run-on assays, by 1.7 and 1.9-fold respectively. Addition of cholera toxin (10 ng/ml) or forskolin (10 nM) stimulated the 3β-HSD-gene transcription by 2.15 and 2.4-fold respectively. We conclude that gonadotropins positively regulate 3β-HSD transcriptional activation and appear to have no effect on the 3β-HSD mRNA stability.

Keywords: 3β-HSD; transcriptional activation; FSH; LH; granulosa cells

Introduction

3β-5-hydroxy-5-ene steroid dehydrogenase (3β-HSD) is a key enzyme in hormonal steroid biosynthesis. 3β-HSD catalyzes the dehydrogenation and isomerization of Δ^5-3β-hydroxysteroids which is an obligatory intermediate in the biosynthesis of progesterone, androgens, estrogens, glucocorticoids and mineralocorticoids. It has been suggested that the expression of 3β-HSD in the ovary is constitutive (Erickson *et al.*, 1986; Gore-Langton & Armstrong 1994). However, our previous studies, conducted in cultured porcine granulosa cells, have indicated that the levels of 3β-HSD mRNA are positively regulated by gonadotropins and stimulators of protein kinase A (PKA) and negatively regulated by activators of protein kinase C (PKC) (Chedrese *et al.*, 1990a,b). Studies conducted in cows have shown that changes in the levels of 3β-HSD mRNA, protein content and enzymatic activity occur during the luteal phase of the estrus cycle (Couet *et al.*, 1990). Taken together the results of these studies suggest that the expression of the 3β-HSD gene is a hormone-regulated step in steroids synthesis within the ovary.

The objective of the present study, conducted in cultured porcine granulosa cells, was to further characterize the effect of FSH and LH on: (1) the steady-state levels of 3β-HSD mRNA, measured by Northern analysis and (2) the rate of newly synthesized 3β-HSD mRNA measured by transcription run-on assays.

Correspondence: P. Jorge Chedrese
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Results

Effect of gonadotropins on the steady-state levels of 3β-HSD mRNA

Figure 1 shows representative Northern analyses of granulosa cells total RNA, demonstrating the effect of FSH (A) or LH (B), followed by FSH- or LH-withdrawal, on the levels of 3β-HSD mRNA. Figure 2 represents the effect of FSH or LH on granulosa cells for 8 h, followed by FSH- and LH-withdrawal and re-addition of FSH (Figure 2A) or LH (Figure 2B), on the levels of 3β-HSD mRNA measured by Northern blot analyses and quantitated by densitometry. FSH or LH elevated the levels of 3β-HSD mRNA to 3.0 and 2.5-fold ($P<0.01$) over the control levels respectively. The withdrawal of FSH or LH induced a rapid decay of these levels, reaching the control values after 2 h incubation. The half life of 3β-HSD mRNA, after FSH- and LH-withdrawal, was estimated to occur in 1 h. The group of granulosa cells washed and immediately treated with FSH (Figure 2A) or (LH Figure 2B) exhibited elevation of the steady-state levels of 3β-HSD mRNA to 4.0 and 3.2-fold ($P<0.01$) respectively, over a subsequent 8 h incubation. Re-addition of FSH (Figure 2A) or LH (Figure 2B) after 4 h of FSH- or LH-withdrawal, elevated the steady-state levels of 3β-HSD mRNA to 4.8 and 5.3-fold ($P<0.01$), respectively.

Figure 3 and Table 1 represent the effect of actinomycin D on the FSH- and LH-induced elevation of the 3β-HSD mRNA levels in granulosa cells. Treatment with actinomycin D, for a period of 6 h, induced a 40% to 50% ($P<0.05$) inhibition in the levels of 3β-HSD mRNA over the control group (Table 1). Treatment of granulosa cells with actinomycin D, before the addition of FSH or LH, inhibited the gonadotropin-stimulated 3β-HSD mRNA levels (Table 1). Addition of actinomycin D to granulosa cells

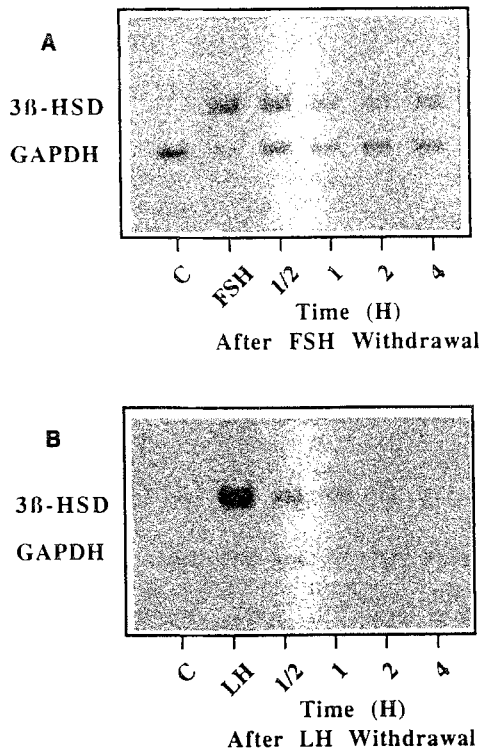

Figure 1 Northern analyses of total RNA from cultured porcine granulosa cells treated with FSH (**A**) or LH (**B**) followed by FSH- or LH-withdrawal. Granulosa cells were cultured in the absence (Control) or presence of FSH or LH (100 ng/ml). After 8 h exposure cells were washed extensively and treated with gonadotropins-free medium. At the indicated periods of time granulosa cells were collected and the RNA was extracted for Northern blot analysis

previously treated with FSH (Figure 3A) or LH (Figure 3B) induced a rapid decay in the levels of 3β-HSD mRNA. These levels reached the control values ($P > 0.05$) after 2 h of actinomycin D exposure. The estimated half-lives of 3β-HSD mRNA after actinomycin D transcription inhibition were 1.3 h and 1.2 for the FSH- and LH-treated groups, respectively.

Effect of gonadotropins on the transcriptional activation of the 3β-HSD-gene

Figure 4 represents the effect of FSH, LH, cholera toxin and forskolin on the transcriptional activation of the 3β-HSD-gene. FSH and LH stimulated transcription of the 3β-HSD-gene by 1.7 and 1.9-fold over the control respectively. Cholera toxin or forskolin, stimulated 3β-HSD-gene transcription rate by 2.15 and 2.4-fold respectively. No changes were observed in the transcription rate of the control gene GAPDH with any of the treatments.

Discussion

In this report we have examined the effect of FSH and LH on the steady-state levels of 3β-HSD mRNA and on the newly synthesized 3β-HSD mRNA in cultured porcine granulosa cells. These experiments were

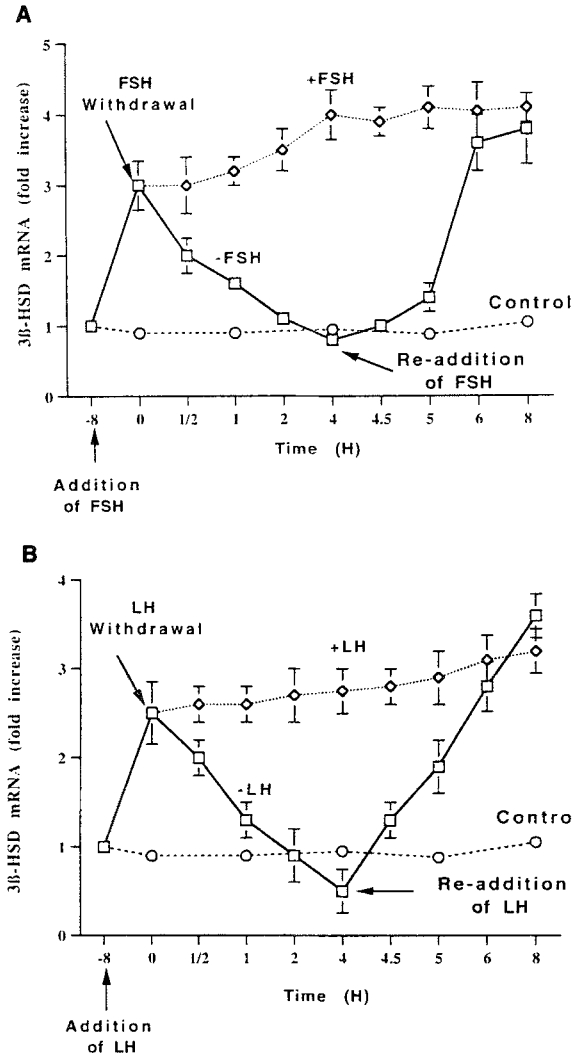

Figure 2 Effect of FSH (**A**) and LH (**B**) withdrawal and re-addition on 3β-HSD mRNA levels in cultured porcine granulosa cells. Triplicate plates of granulosa cells were cultured in the absence (Control) or presence of FSH or LH (100 ng/ml). After 8 h exposure cells were washed extensively and treated with medium with (+FSH and +LH) or without (−FSH and −LH) gonadotropins. In a second group of cells gonadotropins were re-added after 4 h of gonatropin withdrawal. At the indicated periods of time granulosa cells were collected and the RNA was extracted for Northern blot analysis. Data were quantitated by densitometry, as described in Materials and methods. 3β-HSD mRNA levels were corrected for hybridization to GAPDH mRNA. The means ± SEM of three separate experiments are expressed relative to the basal 3β-HSD mRNA level in the control group. The absence of error bars is attributed to a very low dispersion of the data

prompted by the observation that gonadotropins positively regulate 3β-HSD mRNA levels (Chedrese *et al.*, 1990a). We now show that: (1) the withdrawal of gonadotropin from culture media induced a rapid decrease in the steady-states levels of 3β-HSD mRNA and (2) that re-addition of gonadotropins, after a depletion period of 4 h, re-elevated the levels of 3β-HSD mRNA. The present investigation also confirms our previous observations suggesting that the positive effect of gonadotropins on the steady-states levels of 3β-HSD mRNA were mediated through transcriptional activation of the 3β-HSD-gene (Chedrese *et al.*, 1990a).

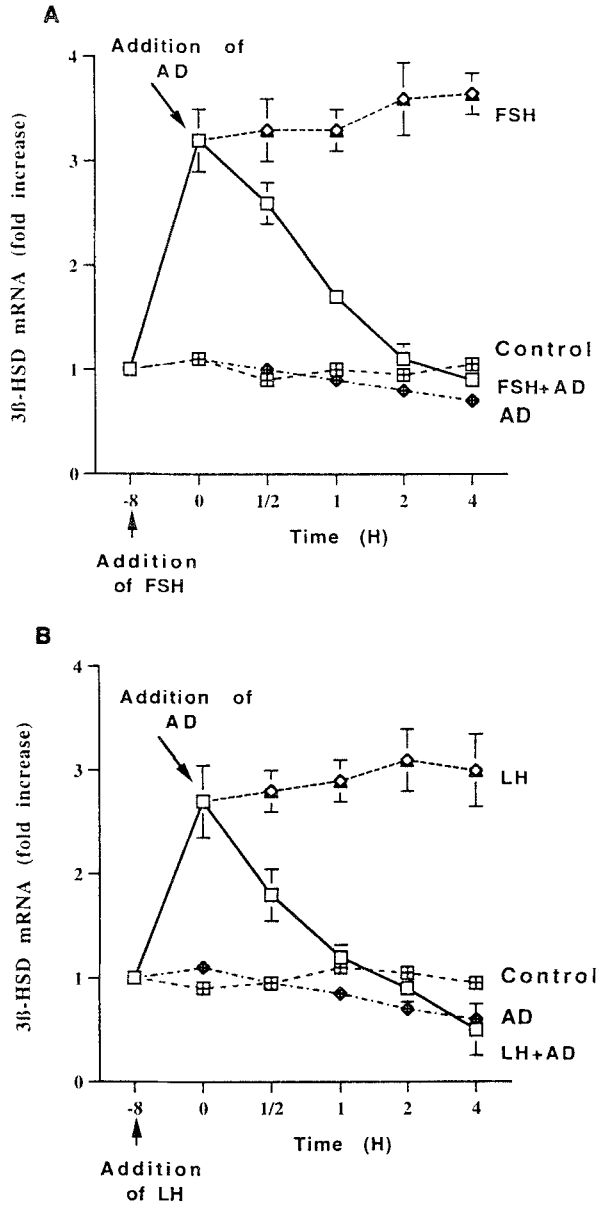

Figure 3 Effect of Actinomycin D on the levels of 3β-HSD after stimulation with FSH (**A**) or LH (**B**). Triplicate plates of granulosa cells were cultured in the absence (Control) or presence of FSH or LH (100 ng/ml). After 8 h incubation actinomycin D, at a final concentration of 1 μg/ml, was added to the cultures. At the indicated periods of time granulosa cells were collected, and analysed as described in the legend to Figure 2. The means ± SEM of three separate experiments are expressed relative to the basal 3β-HSD mRNA level in the control group. The absence of error bars is attributed to a very low dispersion of the data

Table 1 Effect of actinomycin D (AD) on basal, FSH-and LH-stimulated 3β-HSD mRNA levels in cultured porcine granulosa cells

	−AD	*+AD*
Control	1.0 (0.10)	0.5 (0.06)
FSH	3.0 (0.20)	0.7 (0.08)
LH	2.5 (0.15)	0.8 (0.07)

Triplicate plates of granulosa cells were treated with 1 μg/ml actinomycin D (AD) for 15 min and then cultured in the absence (control) or presence of FSH or LH (100 ng/ml). After 6 h incubation granulosa cells were collected and analysed as described in the legend to Figure 1. The means ± SEM of three separate experiments are expressed relative to the basal 3β-HSD mRNA level in the control group

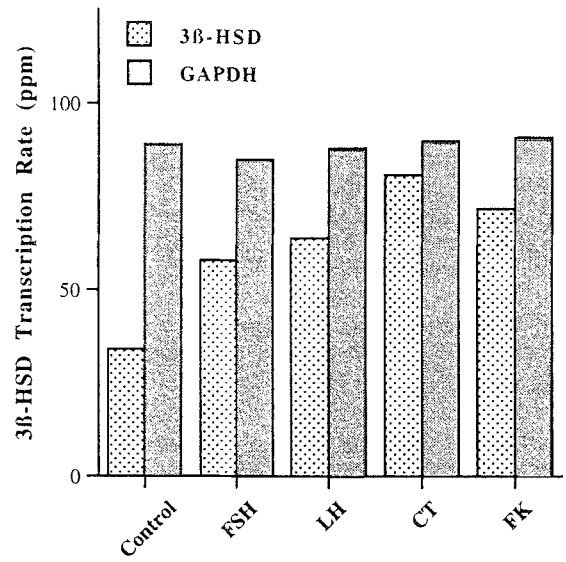

Figure 4 Effect of FSH, LH, cholera toxin (CT) and forskolin (FK) on 3β-HSD-gene transcription. Triplicate plates of granulosa cells were cultured in the absence (Control) or presence of FSH, LH (100 ng/ml), cholera toxin (10 ng/ml) or forskolin (10 nM) for 4 h. After this period of time granulosa cells were collected, pooled and nuclei isolated. Transcription rates were determined by nuclear run-on assays as described in Materials and Methods. Results are expressed in parts per million (ppm) after subtracting background hybridization from the plasmid DNA (pGEM) and corrected for the efficiency of hybridization. Values are the mean of duplicates from one representative experiment

Treatment of cultured granulosa cells with actinomycin D, before the addition of gonadotropins, inhibited the FSH- and LH-induced elevation in the 3β-HSD mRNA levels (Table 1). Addition of actinomycin D, to granulosa cells previously treated with gonadotropins, reduced the levels of 3β-HSD mRNA in a similar fashion as gonadotropins withdrawal. In both cases, the estimated half-lives of 3β-HSD mRNA were very similar. Gonadotropins elevated the levels of newly transcribed 3β-HSD-mRNA as measured by nuclear run-on. Newly transcribed 3β-HSD mRNA levels also were elevated in granulosa cells exposed to the adenylate cyclase activators cholera toxin and forskolin. Taken together the results indicate that cAMP-mediated transcriptional activation of the 3β-HSD-gene was responsible for the elevated steady-states levels of 3β-HSD mRNA observed after gonadotropin stimulation.

3β-HSD is a key enzyme in all steroidogenic tissues, yet little information is available about the regulation of 3β-HSD-gene expression in the ovary. Many studies have been devoted to the regulation of steroid P450 hydroxylases, thus creating a general belief that 3β-HSD does not represent a regulated step in the control of steroid biosynthesis in granulosa cells or in the corpus luteum (Gore-Langton & Armstrong 1994). Our gonadotropin withdrawal and re-addition experiments demonstrated that the expression of the 3β-HSD-gene was highly regulated in porcine granulosa cells (Figure 2A and B). Under these conditions FSH and LH were strict requirements for the expression of the 3β-HSD-gene. The results of the actinomycin D experiments reported herein confirmed these observations and demonstrated that the 3β-HSD mRNA was very labile

after transcription inhibition. Taken together the results may be also interpreted to suggest that stabilization of 3β-HSD-mRNA does not play a substantial role in the gonadotropin regulation of the 3β-HSD-gene in granulosa cells.

The mechanism of transcriptional regulation of the 3β-HSD-gene in the ovary has not been thoroughly investigated. In humans, two types of 3β-HSD genes have been identified. The type I 3β-HSD-gene is almost exclusively transcribed in the mammary gland, placenta and skin (Lachance *et al.*, 1990). The type II 3β-HSD-gene, which displays 93.5% homology with the type I 3β-HSD-gene, is the predominant species transcribed in the adrenals, ovaries and testes (Lachance *et al.*, 1991). This information could be interpreted to suggest that the 3β-HSD-genes may be regulated by tissue specific factor(s) and/or be coordinated with the rest of the steroidogenic enzymes genes in the ovary.

Studies on gene organization of the human type II 3β-HSD have indicated that the putative cAMP binding protein regulatory element (CRE) is located at position −396 (Lachance *et al.*, 1991). CREs have been identified in the promoters of several cAMP responsive genes, along with a group of cognate transcription factors that are members of the CRE-binding protein/activating transcription factor family (Jameson & Hollenberg, 1993). We previously demonstrated that gonadotropin-induced 3β-HSD mRNA levels are dependent on newly synthesized proteins (Chedrese *et al.*, 1990a). Whether a protein transcription factor, binding a 3β-HSD-gene CRE, mediates the gonadotropin-activated transcription reported herein remains to be investigated.

In conclusion, the present investigation, conducted in cultured porcine granulosa cells has demonstrated that: (1) 3β-HSD-gene expression is highly regulated by gonadotropins; (2) gonadotropins mainly affect 3β-HSD-gene transcriptional activation rather than mRNA stabilization and (3) gonadotropin-activated 3β-HSD-gene transcription appears to be mediated through cAMP. We postulate that 3β-HSD-gene transcriptional activation is also one of the mechanisms by which FSH and LH control ovarian progesterone biosynthesis.

Materials and methods

Reagents and hormones

Dulbecco's MEM, antibiotic-antimycotic mixture, fetal calf serum and reagents used for RNA preparations and Northern analyses were obtained from Gibco-BRL (Burlington, Ontario). Forskolin and cholera toxin were purchased from Sigma Chemical Co. (St. Louis, MO). FSH (NIADDK-oFSH) and LH (NIADDK-oLH) were obtained as a gift from National Hormone and Pituitary Program (Rockville, Maryland).

Granulosa cell culture

Ovaries of prepubertal gilts were obtained from a local abattoir. Granulosa cells were collected from medium-sized non-atretic follicles (4 to 6 mm) and washed three times in Dulbecco's MEM containing 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml fungizone (DMEM), as previously described (Chedrese *et al.*, 1987). Viable granulosa cells were plated in 100 mm plastic cell culture plates (Falcon, Lincoln Park, NJ) at a density of 5×10^6 viable cells/well. Cell cultures were maintained in a CO_2 incubator (Forma Scientific Inc. Marietta, Ohio) at 37°C under a water saturated atmosphere of 95% air and 5% CO_2. Cells were initially cultured in serum-containing (10% FCS) DMEM for 48 h to allow attachment onto the plates; then cultured for an additional 48 h in serum-free DMEM, subsequently referred to as culture medium. At the end of this period, the experiments were initiated by replacing the medium with fresh culture medium containing the treatments.

Studies of 3β-HSD mRNA decay rates

In a first set of experiments the decay rate of 3β-HSD mRNA was studied by stimulating granulosa cells with gonadotropins, followed by withdrawal of the gonadotropins. Granulosa cells were cultured in the absence (control) or presence of FSH or LH at a concentration of 100 ng/ml. After 8 h incubation, the control group was washed with culture medium without gonadotropins and the gonadotropin treated cells were divided into three groups. The first was washed with culture medium with gonadotropins; the second was washed with culture medium without gonadotropins; and the third was washed with culture medium without gonadotropins, followed by a re-addition of gonadotropins after a 4 h incubation. Granulosa cells were incubated for 0.5, 1, 2, 4, 4.5, 5, 6, and 8 h.

In a second set of experiments, the decay of gonadotropin-induced 3β-HSD-mRNA steady-state levels was investigated using the transcription inhibitor actinomycin D. Granulosa cells were cultured in the absence or presence of FSH or LH (100 ng/ml). Cells were treated with actinomycin D (1 μg/ml) after 8 h incubation and re-incubated for 0.5, 1, 2 and 4 h. The concentration of actinomycin D used was selected from a previous experiment in which the minimum amount capable of inhibiting LH-induced 3β-HSD mRNA levels, without affecting granulosa cell viability, was determined.

Northern blot analyses

Granulosa cells, from triplicate plates, were collected using 2 ml of 1% sodium dodecyl sulphate- (SDS) 10 mM EDTA pH 7.0 solution and RNA was isolated by acid phenol/chloroform extraction (Liu *et al.*, 1994). Samples of total RNA (5 μg) were denatured, size-fractionated by electrophoresis on a 1% agarose-formaldehyde gel and transferred onto a nylon membrane (Hybond-N, Amersham Canada Ltd. Oakville, Ontario) by diffusion blotting. RNA was crosslinked to membrane using a UV Stratalinker 1800 (Stratagene, La Jolla, CA). cDNAs complementary to mRNAs encoding 3β-HSD (Luu-The *et al.*, 1989) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Tso *et al.*, 1985) were used as probes. cDNAs were labeled by random primer synthesis (Feinberg & Vogelstein 1983) with [α^{32}P]dCTP (>3000 Ci/mmol; New England Nuclear, Boston, MA) to a specific activity of $1.5-3.0 \times 10^9$ dpm/μg DNA. Membranes were hybridized for 16 h at 65°C in a solution containing 1 M Na Cl, 10% dextran sulfate and 1% SDS. After hybridization, membranes were washed twice for 15 min at room temperature in 2 × SSC-0.5% SDS and twice in 1 × SSC−0.5% SSC at 65°C (20 × SSC contained 3 M NaCl and 0.3 M Na_3 citrate). Hybridizations were first performed with labeled 3β-HSD cDNA. Radioactive labeling were removed by incubating the membranes in 10 mM Tris-10 mM EDTA for 30 min at 90°C before probing with labeled GAPDH cDNA. Northern blot autoradiograms were quantitated by computer-aided scanning densitometry using a ScanJet IIp Hewlett Packard scanner and analysed with a digital image processing program (NIH Image 1.41). Data were corrected for variability in loading by calculation as ratio to GAPDH, which was unaffected by treatment with FSH or LH.

Transcription run-on assays

Granulosa cells were cultured in the absence or presence of FSH, LH (100 ng/ml), cholera toxin (10 ng/ml) or forskolin (10 nM). After 4 h incubation granulosa cells were collected and homogenized in 10 mM Tris-Cl, pH 7.4, 3 mM $CaCl_2$, 3 mM $MgCl_2$, 0.5% Nonidet P-40 using a Dounce homogenizer. Nuclei were collected by centrifugation at 500 *g* for 10 min and resuspended in 200 µl of storage buffer (50 mM Tris-Cl pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA and maintained in liquid nitrogen.

Newly synthesized mRNA transcripts were analysed using a modification of the procedure described by McKnight and Palmiter (1979). Isolated nuclei (5×10^7 nuclei in 200 µl) were mixed with 200 µl of transcription buffer (10 mM Tris-Cl pH 8, 5 mM $MgCl_2$, 0.3 KCl) containing 0.5 mM each of ATP, GTP and CTP and 100 µCi [α-^{32}P]UTP (3000 Ci/mmol, New England Nuclear). After incubation at 30°C for 30 min, labeled RNA was isolated by digestion with DNAse I (35 U) and proteinase K (100 µg) followed by extraction in 1% SDS and acid phenol (Liu *et al.*, 1994) using 10 µg *E. coli* tRNA carrier. Labeled RNA ($\sim 5 \times 10^7$ cpm) was hybridized to membranes at 65°C for 72 h under the conditions described above. Membranes contained excess (5 µg) of 3β-HSD or GAPDH cDNAs. The pGEM-3 cloning vector DNA was used as a control for background hybridization. We performed DNA excess filter hybridization to estimate the hybridizable radioactivity in 3β-HSD mRNA as described previously (Chedrese *et al.*, 1994). Radioactivity in the membranes was quantified by liquid scintillation spectrometry. Synthesis rates for mRNAs were calculated from the radioactivity values of [^{32}P]RNA bound to specific cDNA-containing membranes minus the value of pGEM-3 containing membranes. The data were expressed as part per million (ppm) for the 3β-HSD-gene transcription after correction for the efficiency of hybridization.

Statistical analyses

Northern blot analyses data were subjected to two-way analysis of variance. In the presence of significant F values, means were compared using Duncan's multiple range test (Steele & Torrie 1980).

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