Genetic differences in Follicular DNA Synthesis between Two Strains of Hamsters

(43069)

SHYAMAL K. ROY AND GILBERT S. GREENWALD[1]

Department of Physiology, Ralph L. Smith Research Center, University of Kansas Medical Center, Kansas City, Kansas 66103

Abstract. **This study was designed to compare our previous results on ovarian follicular DNA synthesis by hamsters obtained from Sasco Laboratories with a different breeding colony: Harlan. Follicles from proestrous Harlan hamsters required twice as much [^{3}H] thymidine and a minimum of 4 hr of *in vitro* exposure to 100 ng of ovine follicle-stimulating hormone (FSH) before a significant increase in DNA synthesis was elicited compared with 30–120 min for the Sasco breed. Peak responsiveness to FSH was observed at 8-hr incubation for the Harlan strain with significant increases in DNA per follicle at 8–12 hr. Both strains increased DNA synthesis with as little as 25 ng of ovine FSH and the response was elicited in all growing follicles, from preantral stages with one to four layers of granulosa cells, lacking theca (Stages 1–4) to mature antral follicles (Stages 8–10). A recombinant bovine FSH, devoid of luteinizing hormone activity, was not as effective as ovine FSH (which has 4% luteinizing hormone contamination) in stimulating DNA synthesis by large preantral and antral follicles. *In vitro* responsiveness to ovine FSH was abolished in the absence of Ca^{2+} in the culture medium and 0.05 m*M* Ca^{2+} was the optimal amount. For both strains of hamsters, the highest rate of DNA synthesis in response to endogenous gonadotropins was on the morning of estrus—when the second surge of FSH was in progress—and Harlan follicles *in vitro* also showed maximal stimulation by FSH on this day. Where the two strains differed was that the Harlan strain did not show an increase in follicular DNA synthesis on the afternoon of proestrus—when the preovulatory increase in gonadotropins commenced. When expressed as DNA per follicle, DNA approximately doubled from Stages 1 to 5 and then entered a new growth phase at Stage 6 (large preantral follicles) with a steeper increase. Collectively, these experiments show that strain characteristics can alter the latency and degree of follicular DNA replication in response to endogenous or exogenous FSH.** [P.S.E.B.M. 1990, Vol 194]

In a series of *in vivo* and *in vitro* studies, we have shown that hamster preantral follicles respond to follicle-stimulating hormone (FSH) by significant increases in DNA and progesterone synthesis (for references, see 1). These studies were carried out with animals purchased from Sasco Laboratories (Omaha, NE), the source of hamsters used in all our experiments over the past 15 years. The most recent investigation revealed that *in vitro* exposure to ovine FSH for 2 hr elicited significant increases in DNA synthesis in preantral and antral follicles from proestrous Sasco-derived hamsters (1).

In 1986, Sasco Laboratories phased out their original hamster colony to replace it with a "hardier" virus-free stock derived from animals maintained for 20 years as an inbred colony by the Epley Cancer Center, University of Nebraska (Omaha). To our consternation, the *in vitro* results reported previously for the old stock (1) were no longer reproducible. The present experiments were therefore designed to determine what were the variables involved in the initial inability to repeat the results with the new Sasco colony. The hamsters used in the definitive experiments were obtained from a different source (Harlan, Madison, WI) and the data presented herein also therefore serve as the baseline for future studies which will utilize this colony.

[1] To whom all correspondence and requests for reprints should be addressed.

Received October 23, 1989. [P.S.E.B.M. 1990, Vol 194]
Accepted February 21, 1990.

0037-9727/90/1942-0136$2.00/0

Materials and Methods

Ovine FSH-17 and ovine LH-25 were obtained from the National Pituitary Hormone Program (NIH); recombinant bovine FSH was a gift from Dr. Scott Chappel, Department of Molecular Biology, Integrated Genetics, Inc., Framingham, MA); [^{3}H]thymidine ([^{3}H]TdR) (sp act, 21 Ci/mmol) was obtained from Amersham (Arlington Heights, IL); and calf thymus DNA and bisbenzimide (Hoeschst dye 33528) were purchased from Sigma Chemical Co. (St. Louis, MO). All other analytical grade reagents were purchased from various commercial sources.

Adult golden hamsters (90–100 g) were obtained from Harlan Laboratories and at least three consecutive 4-day cycles were monitored by the conspicuous postovulatory vaginal discharge on estrus (Day 1 of the cycle) before the animals were used. Day 4 corresponds to proestrus. All animals were sacrificed at 0900 hr and the ovaries collected in cold (4°C) Krebs-Ringer solution supplemented with bicarbonate and 25 m*M* Hepes (KRBH; pH 7.2). Large preantral and antral follicles (Stages 6–10) were dissected under a stereoscope and smaller follicles (Stages 1–5) were dissociated enzymatically, as described previously (2). The stages of development are defined as follows: Stages 1–4, follicles with one to four layers of granulosa cells and no theca; Stages 5 and 6, follicles with five and six and seven and eight layers of granulosa cells and developing theca, respectively; Stage 7, follicles with beginning antrum; and Stages 8–10, small, intermediate, and large antral follicles (2).

Follicles at Stages 1–10 of development were incubated in 700 μl of KRBH with 0.2% bovine serum albumin for 2 to 12 hr at 37°C in air in the presence of 1 μCi of [^{3}H]thymidine ([^{3}H]TdR) with or without ovine FSH or luteinizing hormone (LH) in a shaking water bath. There were four replicates per treatment group, each one consisting of 100 follicles for Stage 1, 50 for Stages 2–4, 30 for Stage 5, 15 for Stage 6, 10 for Stage 7, 4 for Stage 8, 2 for Stage 9, and 3 for Stage 10. At the end of incubation, follicles were separated, washed twice in DNA assay buffer, pH 7.0 (3), and stored at −20°C until assayed. Follicular DNA content and incorporation of radionucleotide into DNA were measured by fluorometry and scintillation counting, respectively, as described previously (3).

The results for follicular thymidine incorporation were expressed as picomoles per microgram of DNA. The data were analyzed by analyses of variance and Duncan's multiple range test with the level of significance established at 5%.

Results

Experiments with the New Stock of Sasco Hamsters. When follicles at Stages 1–10 were collected on proestrus (Day 4) and estrus, during the periods of periovulatory increases of FSH and LH, incubation for 2 hr failed to increase DNA synthesis above the rate of incorporation into follicles collected at Day 4, 0900 hr (several hours before the surge in gonadotropins). Similarly, incubation for 2 hr of early proestrous follicles (0900 hr) with 25 ng of FSH failed to increase DNA synthesis (values similar to control in Table II).

In the next experiment, ovaries were removed at Day 4 (0900 hr) and Stages 1–10 follicles were isolated and incubated for 4 hr with [^{3}H]TdR in the absence or presence of 100 ng of FSH or 25 ng of recombinant FSH. Under these circumstances, there was an extraordinary 5- to 8-fold increase in DNA synthesis in the control group compared with a 2-hr control but no significant increase above these elevated values by either FSH treatment. Based on these and other negative results with the new Sasco colony, attention was shifted to using animals from a different source: Harlan Laboratories, which were used in all subsequent experiments.

Effects of Different Doses of Ca^{2+} on DNA Synthesis by Proestrous Follicles. In the absence of Ca^{2+}, incubation for a total of 8 hr with 100 ng of FSH was ineffective in stimulating DNA synthesis in the last 4 hr of incubation with [^{3}H]TdR, whereas 0.05 m*M* Ca^{2+} was the most effective dose (Table I). With higher amounts of Ca^{2+}, FSH was less effective. Krebs-Ringer-bicarbonate normally contains 1.8 m*M* Ca^{2+}. When control follicles were incubated for 8 hr with this amount of Ca^{2+}, DNA synthesis was reduced for Stages 1 to 5 from incubation with 0.05 m*M*: for Stage 1, a reduction of 39%; Stage 2, 20%; Stage 3, 22%; Stage 4, 48%; and Stage 5, 30%. Hence, 0.05 m*M* Ca^{2+} was used with KRBH in all subsequent experiments. When various nucleotides were added to the medium along with 0.05 m*M* Ca^{2+}, the ability of FSH to stimulate DNA synthesis was lost (Table I).

Temporal Effect of 100 ng of FSH on Follicular DNA Synthesis. Unlike our previous experience with Sasco hamsters, DNA synthesis was not detectable in follicles from Harlan hamsters until 4 hr of incubation with a peak reached at 8 hr, followed by a plateau in activity at 12 hr (Table II). It is noteworthy that follicles incubated without FSH showed a temporally related increase in [^{3}H]thymidine incorporation as one proceeded from time 0 to 12 hr of incubation. Follicular DNA began to show significant increases in response to FSH by 8–12 hr (Table III).

Effects of Graded Doses of FSH and LH on Follicular DNA Synthesis. With a 4-hr exposure to [^{3}H]TdR during a total incubation of 8 hr, DNA synthesis was significantly increased by 25 ng of FSH with no dose-response relationship evident with higher doses of FSH (Table IV). Ovine FSH has a 4% contamination with LH and hence the effects of 5 ng of LH were compared with those of 100 ng of FSH. Under these

Table I. Effects of *In Vitro* Ca^{2+} on FSH (100 ng/ml) Induced DNA Replication of Proestrous Hamster Follicles

Ca^{2+} (m*M*)	Group	[^{3}H]TdR (pmol/μg DNA/4 hr)[a]									
		Stages of follicle									
		1	2	3	4	5	6	7	8	9	10
0	Control	0.3 ± 0.03	0.3 ± 0.004	0.31 ± 0.02	0.2 ± 0.02	0.05 ± 0.01	0.12 ± 0.02	0.14 ± 0.02	0.07 ± 0.01	0.02 ± 0.002	0.13 ± 0.04
	FSH	0.4 ± 0.04	0.32 ± 0.01	0.30 ± 0.01	0.06 ± 0.005	0.02 ± 0.004	0.08 ± 0.007	0.13 ± 0.03	0.10 ± 0.01	0.02 ± 0.002	0.08 ± 0.008
0.05	Control	0.3 ± 0.007	0.44 ± 0.02	0.43 ± 0.02	0.17 ± 0.005	0.18 ± 0.005	0.09 ± 0.001	0.06 ± 0.009	0.12 ± 0.003	0.01 ± 0.001	0.13 ± 0.004
	FSH	0.8 ± 0.04	0.63 ± 0.05	0.84 ± 0.04	0.30 ± 0.02	0.30 ± 0.03	0.26 ± 0.03	0.21 ± 0.006	0.16 ± 0.005	0.02 ± 0.001	0.17 ± 0.001
0.50	Control	0.22 ± 0.005	0.24 ± 0.01	0.36 ± 0.02	0.08 ± 0.004	0.14 ± 0.006	0.12 ± 0.001	0.14 ± 0.004	0.13 ± 0.004	0.01 ± 0.001	0.11 ± 0.02
	FSH	0.38 ± 0.02	0.40 ± 0.02	0.45 ± 0.02	0.15 ± 0.01	0.08 ± 0.01	0.10 ± 0.007	0.12 ± 0.007	0.06 ± 0.006	0.008 ± 0.001	0.10 ± 0.01
2.5	Control	0.41 ± 0.01	0.56 ± 0.07	0.40 ± 0.02	0.16 ± 0.009	0.25 ± 0.02	0.13 ± 0.007	0.07 ± 0.008	0.03 ± 0.004	0.01 ± 0.001	0.08 ± 0.002
	FSH	0.40 ± 0.04	0.55 ± 0.04	0.66 ± 0.02	0.30 ± 0.01	0.35 ± 0.01	0.27 ± 0.02	0.14 ± 0.01	0.15 ± 0.01	0.009 ± 0.001	0.10 ± 0.01
0.05 + nucleotides[b]	Control	0.31 ± 0.03	0.31 ± 0.02	0.28 ± 0.02	0.13 ± 0.006	0.07 ± 0.007	0.09 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.01 ± 0.002	0.09 ± 0.02
	FSH	0.34 ± 0.03	0.40 ± 0.02	0.49 ± 0.03	0.09 ± 0.006	0.07 ± 0.02	0.12 ± 0.004	0.07 ± 0.003	0.09 ± 0.01	0.02 ± 0.001	0.06 ± 0.004

[a] [^{3}H]TdR (1 μCi/ml) was added 4 hr before termination of an 8-hr incubation in this experiment. Ca^{2+} ions were supplied as $CaCl_2$ in Krebs-Ringer salt solution. Underlined values are significantly ($P < 0.05$) different from respective controls.
[b] ATP, GTP, and CTP were supplied at concentrations (100 m*M*) similar to Ham's F-12.

Table II. Temporal Effects of FSH (100 ng/ml) *In Vitro* on Follicular DNA Replication[a]

Hr	Group	[^{3}H]TdR (pmol/μg DNA)									
		Stages of follicle									
		1	2	3	4	5	6	7	8	9	10
2[b]	Control	0.08 ± 0.008	0.12 ± 0.005	0.14 ± 0.01	0.20 ± 0.02	0.15 ± 0.02	0.07 ± 0.02	0.16 ± 0.02	0.15 ± 0.02	0.005 ± 0.002	0.09 ± 0.004
	FSH	0.08 ± 0.006	0.12 ± 0.02	0.14 ± 0.01	0.13 ± 0.01	0.09 ± 0.007	0.14 ± 0.04	0.14 ± 0.03	0.15 ± 0.02	0.006 ± 0.002	0.15 ± 0.01
4[c]	Control	0.25 ± 0.01	0.30 ± 0.03	0.41 ± 0.04	0.26 ± 0.02	0.34 ± 0.04	0.44 ± 0.04	0.24 ± 0.02	0.17 ± 0.02	0.01 ± 0.001	0.21 ± 0.02
	FSH	0.40 ± 0.02	0.31 ± 0.03	0.30 ± 0.02	0.23 ± 0.02	0.22 ± 0.03	0.32 ± 0.03	0.30 ± 0.12	0.16 ± 0.02	0.01 ± 0.003	0.23 ± 0.03
8[d]	Control	0.62 ± 0.01	0.21 ± 0.01	0.21 ± 0.03	0.16 ± 0.02	0.10 ± 0.01	0.14 ± 0.008	0.10 ± 0.005	0.10 ± 0.005	0.01 ± 0.003	0.09 ± 0.01
	FSH	2.2 ± 0.03	1.9 ± 0.08	0.91 ± 0.04	0.70 ± 0.03	0.50 ± 0.03	0.43 ± 0.01	0.43 ± 0.01	0.26 ± 0.02	0.23 ± 0.02	0.24 ± 0.009
12[d]	Control	3.1 ± 0.15	2.1 ± 0.12	1.4 ± 0.09	0.80 ± 0.08	0.67 ± 0.07	0.35 ± 0.03	0.40 ± 0.03	0.43 ± 0.02	0.41 ± 0.04	0.27 ± 0.02
	FSH	2.3 ± 1.7	2.2 ± 0.08	1.2 ± 0.05	0.80 ± 0.06	0.34 ± 0.03	0.42 ± 0.02	0.42 ± 0.02	0.44 ± 0.07	0.30 ± 0.009	0.44 ± 0.05

[a] Underlined values are significantly ($P < 0.05$) different within each stage from control values.
[b] Incubated for 2 hr with [^{3}H]TdR.
[c] Incubated for 4 hr with [^{3}H]TdR.
[d] [^{3}H]TdR (1 μCi/ml) was added 4 hr before termination of an 8- or 12-hr incubation. Krebs-Ringer medium contained 0.05 m*M* Ca^{2+}.

Table III. Follicular DNA Content Following *In Vitro* Incubation in the Absence or Presence of FSH (100 ng/ml)[a]

Hr	Group	DNA (ng/follicle)									
		Stages of follicle									
		1	2	3	4	5	6	7	8	9	10
2	Control	0.5 ± 0.07	0.9 ± 0.1	2 ± 0.1	3 ± 0.1	5 ± 0.7	16 ± 2	52 ± 7	120 ± 11	252 ± 19	706 ± 70
	FSH	0.4 ± 0.03	1 ± 0.07	2 ± 0.1	3 ± 0.4	6 ± 0.4	26 ± 7	70 ± 17	118 ± 11	201 ± 22	705 ± 20
4	Control	0.6 ± 0.05	1 ± 0.2	2.1 ± 0.3	8 ± 0.4	14 ± 0.7	73 ± 5	149 ± 6	209 ± 14	308 ± 32	864 ± 64
	FSH	0.5 ± 0.05	1 ± 0.1	2.3 ± 0.2	6 ± 0.4	13 ± 0.8	62 ± 3	123 ± 12	214 ± 6	400 ± 24	828 ± 61
8	Control	0.84 ± 0.03	2 ± 0.2	3.2 ± 0.4	7 ± 0.5	16 ± 1	61 ± 6	111 ± 11	161 ± 14	322 ± 20	647 ± 29
	FSH	0.74 ± 0.04	2 ± 0.02	3.1 ± 0.05	4 ± 0.6	14 ± 0.7	62 ± 5	125 ± 9	190 ± 24	363 ± 13	808 ± 20
12	Control	0.90 ± 0.06	2.1 ± 0.1	3.3 ± 0.1	5 ± 0.8	15 ± 2	88 ± 7	142 ± 5	181 ± 4	342 ± 34	778 ± 33
	FSH	1.5 ± 0.05	3 ± 0.06	5.0 ± 0.04	6 ± 0.5	20 ± 2	86 ± 2	150 ± 11	206 ± 22	446 ± 22	894 ± 18

[a] Underlined values are significantly ($P < 0.05$) different within each stage.

Table IV. Effects of FSH and LH *In Vitro* on Follicular DNA Synthesis

Group	[^{3}H]TdR (pmol/μg DNA/4 hr)[a]									
	Stages of follicle									
	1	2	3	4	5	6	7	8	9	10
Control	0.37 ± 0.008	0.36 ± 0.004	0.25 ± 0.01	0.20 ± 0.007	0.14 ± 0.008	0.11 ± 0.006	0.09 ± 0.002	0.09 ± 0.008	0.02 ± 0.003	0.04 ± 0.004
FSH (25 ng)	0.53 ± 0.02	0.55 ± 0.04	0.42 ± 0.03	0.26 ± 0.02	0.31 ± 0.02	0.18 ± 0.005	0.08 ± 0.008	0.10 ± 0.007	0.03 ± 0.002	0.08 ± 0.006
FSH (50 ng)	0.66 ± 0.05	0.52 ± 0.02	0.43 ± 0.04	0.30 ± 0.03	0.31 ± 0.03	0.20 ± 0.009	0.07 ± 0.06	0.06 ± 0.006	0.03 ± 0.004	0.03 ± 0.002
FSH (100 ng)	0.45 ± 0.01	0.66 ± 0.04	0.34 ± 0.03	0.33 ± 0.01	0.30 ± 0.03	0.17 ± 0.01	0.15 ± 0.01	0.10 ± 0.002	0.05 ± 0.005	0.09 ± 0.001
LH (5 ng)	0.42 ± 0.04	0.44 ± 0.01	0.31 ± 0.02	0.10 ± 0.01	0.28 ± 0.02	0.06 ± 0.003	0.09 ± 0.005	0.09 ± 0.008	0.02 ± 0.0005	0.06 ± 0.003
FSH (100 ng) + LH (5 ng)	0.76 (2)	0.58 ± 0.02	0.36 ± 0.02	0.17 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.04 ± 0.008	0.03 ± 0.003	0.30 ± 0.002	0.03 ± 0.003

[a] [^{3}H]TdR was added the last 4 hr of a total of 8 hr of incubation. Underlined values are significantly ($P < 0.05$) different within each stage compared with the corresponding control values ($n = 4$).

circumstances, 5 ng of LH was only sporadically effective in increasing DNA synthesis for only three stages of follicular development (Table IV). Paradoxically, 5 ng of LH added along with 100 ng of FSH essentially negated the normal stimulatory action of FSH on DNA synthesis (Table IV).

In a further effort to eliminate LH contamination as a factor in the stimulatory effects of FSH, a recombinant FSH preparation, with no LH activity, was next tested (4). As shown in Figure 1, after an 8-hr incubation with 100 ng of recombinant bovine FSH, in the last 4 hr of exposure to [^{3}H]TdR, follicular DNA synthesis was stimulated for most stages.

Follicular DNA Synthesis throughout the Estrous Cycle. As a follow-up to a previous study with Sasco hamsters (3), Harlan hamsters were sacrificed at 0900 hr for each day of the 4-day estrous cycle and Stages 1–10 follicles were incubated for a total of 8 hr (with [^{3}H] TdR again added the last 4 hr) in the absence or presence of 100 ng of FSH; the latter end point was not previously tested with Sasco hamsters. For control follicles from Stages 1 to 7, the highest rates of incorporation were encountered on Day 1 (estrus) with a gradual subsidence thereafter to the lowest value on the morning of Day 4 (proestrus) (Table V). On the morning of Day 1, the larger sized follicles (Stages 9 and 10) were absent; Stage 8 follicles were just beginning to grow with higher rates of DNA synthesis on Days 2 and 3 as they gradually developed into Stages 9 and 10. Correlating with the [^{3}H]TdR patterns of the control follicles, *in vitro* exposure to FSH led to the highest rates of DNA synthesis on Day 1 with lower rates of incorporation on subsequent days of the cycle (Table VI). Note that on Day 4 Stage 10 follicles, the group destined to ovulate early next morning and which were fully differentiated, no longer responded to FSH.

Contrary to our previous experience with Sasco hamsters, for Harlan hamsters there was no increase in follicular DNA synthesis during the proestrous surge of gonadotropins on the afternoon and evening of Day 4 (Table VI). However, this was followed on estrus (Day 1), when the secondary surge of FSH was in progress, by significant increases in DNA synthesis. This agrees with the results in Table V where significantly higher rates of incorporation of [^{3}H]TdR were evident in the control groups on Day 1 compared with Day 4.

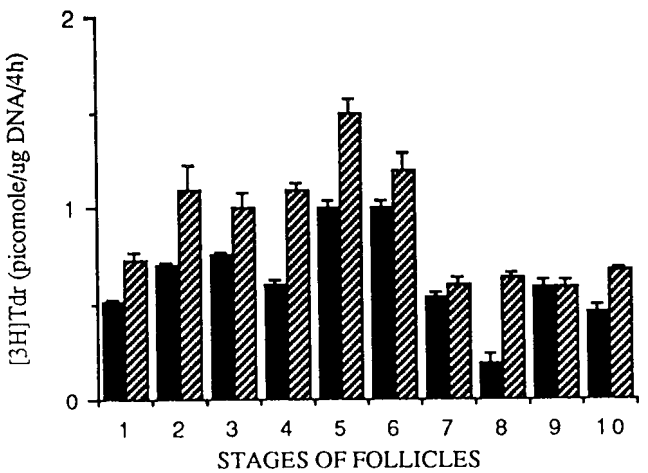

Figure 1. Effects of 100 ng of recombinant bovine FSH (diagonal bars) versus control incubations (solid bars) on [^{3}H]TdR incorporation. There are four replicates for each stage.

DNA Content of Proestrous Hamster Follicles. In the course of these experiments, control groups of follicles were incubated with every treatment regimen and an interesting pattern for DNA per follicle was discernible when average content per follicular stage was calculated. For Stages 1 to 5, the amount of DNA per follicle approximately doubled from one stage to another (Fig. 2). A different accelerated growth phase was evident beginning at Stage 6 when thecal cells began to develop around six and seven layers of granulosa cells (2). Note for Stages 6 to 10, the fairly consistent doubling time as one progressed from large preantral follicles (Stages 6 and 7) to mature preovulatory follicles (Stage 10).

Discussion

The present studies indicate that for the inbred hamster colony, changes in strain characteristics can alter the latency and degree of DNA replication in response to growth stimuli. Although the duration of the estrous cycle (4 days) and the number of ovulations was unchanged in the new "virus-antibody free" hamsters from Sasco, increases in follicular DNA synthesis could not be detected using [^{3}H]TdR either *in vivo* or *in vitro* at any time of the cycle. To maintain cyclicity, new cohorts of preovulatory follicles are recruited from the immediate preantral pool which in turn is replenished by growing small preantral follicles; all of these processes require DNA synthesis and cell proliferation (5). The reason for the inability to detect the time of increased ovarian cell proliferation in the new strain of Sasco hamsters is an enigma; however, the higher basal rate of DNA replication in follicular cells may indicate a shorter G1 phase or cell cycle time. It is also possible that longer times of incubation with [^{3}H]TdR might have yielded positive results.

Utilizing animals from another source (Harlan), however, our previous findings were corroborated and extended, although the Sasco animals showed greater *in vitro* sensitivity to FSH, responding in less than 2 hr (1) compared with 4 hr. Thus, with the Harlan hamsters there is a shift in the window of responsiveness and sensitivity of follicles.

In the absence of Ca^{2+}, addition of 100 ng of FSH did not increase DNA synthesis; 0.05 m*M* Ca^{2+} was the optimal dose. *In vitro* exposure to FSH results in a significant increase in follicular cAMP and 8-bromo-

Table V. Effects of FSH (100 ng/ml) *In Vitro* on [^{3}H]Thymidine Incorporation into Hamster Follicles during the Estrous Cycle

Day of estrous	Group	[^{3}H]TdR (pmol/μg DNA/4 hr)[a] Stages of follicles									
		1	2	3	4	5	6	7	8	9	10
1	Control	0.62 ± 0.02	0.98 ± 0.05	1.1 ± 0.03	0.96 ± 0.03	1.3 ± 0.06	1.3 ± 0.06	0.87 ± 0.02	0.57 ± 0.006		
	FSH	1.0 ± 0.03	1.1 ± 0.008	1.2 ± 0.03	1.1 ± 0.05	1.6 ± 0.07	1.7 ± 0.07	1.2 ± 0.05	1.0 ± 0.05		
2	Control	0.60 ± 0.01	0.74 ± 0.08	0.70 ± 0.04	0.55 ± 0.03	0.55 ± 0.03	0.80 ± 0.01	0.71 ± 0.03	0.79 ± 0.04	0.63 ± 0.02	
	FSH	0.6 ± 0.02	0.87 ± 0.06	0.90 ± 0.06	0.77 ± 0.05	1.2 ± 0.08	1.2 ± 0.08	1.0 ± 0.02	0.70 ± 0.04	0.76 ± 0.04	
3	Control	0.40 ± 0.006	0.72 ± 0.03	0.75 ± 0.02	0.42 ± 0.004	0.47 ± 0.04	0.70 ± 0.02	0.92 ± 0.05	0.67 ± 0.04	0.75 ± 0.05	0.48 ± 0.04
	FSH	0.60 ± 0.02	0.88 ± 0.02	1.0 ± 0.02	0.73 ± 0.02	0.65 ± 0.02	1.2 ± 0.04	0.78 ± 0.04	0.92 ± 0.02	0.61 ± 0.05	0.64 ± 0.03
4	Control	0.36 ± 0.02	0.74 ± 0.02	0.62 ± 0.02	0.50 ± 0.02	0.96 ± 0.04	0.72 ± 0.03	0.51 ± 0.01	0.31 ± 0.02	0.03 ± 0.003	0.42 ± 0.03
	FSH	0.54 ± 0.04	0.84 ± 0.003	1.1 ± 0.002	0.80 ± 0.03	1.2 ± 0.04	1.2 ± 0.05	0.80 ± 0.03	0.50 ± 0.04	0.03 ± 0.008	0.50 ± 0.02

[a] [^{3}H]TdR was added 4 hr before termination of an 8-hr incubation. Underlined values are significantly ($P < 0.05$) different from respective control ($n = 4$).

Table VI. *In Vitro* Incorporation of [^{3}H]Thymidine in Hamster (Harlan Strain) Ovarian Follicles during the Periovulatory Period

Group	[^{3}H]TdR (pmol/μg DNA/4 hr)[a] Stages of follicle									
	1	2	3	4	5	6	7	8	9	10
Day 4: 0900 hr	0.21 ± 0.03	0.29 ± 0.03	0.32 ± 0.03	0.30 ± 0.02	0.30 ± 0.03	0.30 ± 0.02	0.16 ± 0.02	0.13 ± 0.02	0.03 ± 0.002	0.14 ± 0.02
Day 4: 1600 hr	0.24 (2)	0.32 ± 0.07	0.29 ± 0.03	0.30 ± 0.02	0.37 ± 0.03	0.38 ± 0.06	0.25 ± 0.05	0.14 ± 0.02	0.03 ± 0.008	0.15 ± 0.02
Day 4: 2000 hr	0.42 ± 0.02	0.35 ± 0.02	0.30 ± 0.01	0.29 ± 0.03	0.26 ± 0.02	0.37 ± 0.03	0.31 ± 0.04	0.18 ± 0.02	0.03 ± 0.003	0.06 ± 0.005
Day 1: 0900 hr	1.1 ± 0.08	0.93 ± 0.06	0.58 ± 0.03	0.43 ± 0.03	0.43 ± 0.01	0.31 ± 0.07	0.38 ± 0.06	0.38 ± 0.006	—	—
Day 1: 1500 hr	2.2 ± 0.11	1.6 ± 0.08	0.96 ± 0.01	0.71 ± 0.08	0.41 ± 0.03	0.31 ± 0.03	0.18 ± 0.02	0.15 ± 0.02	—	—

[a] Follicles were incubated in KRBH containing 0.05 m*M* Ca^{2+} and 1 μCi of [^{3}H]TdR for a total of 4 hr. No healthy large antral follicles were present on Day 1. Underlined values are significantly ($P < 0.05$) different within each stage. $n = 4$ unless otherwise stated in parentheses.

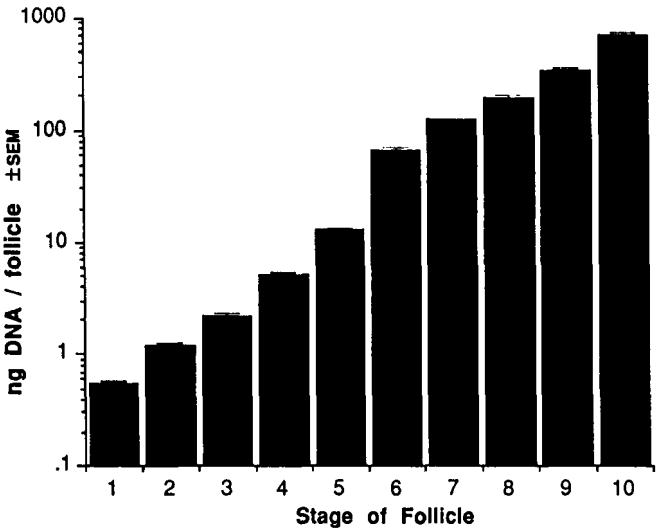

Figure 2. Amount of DNA per stage of follicular development for proestrous (0900 hr) Harlan hamsters. There are 19–23 replicates per stage. Note the logarithmic scale for the DNA values.

cAMP can be substituted for FSH to increase DNA synthesis (1). The fact that external Ca^{2+} is necessary for the stimulatory effects of FSH suggests that protein kinase A may be the major second messenger pathway in follicular DNA synthesis. Tomooka *et al.* (6) have shown that *in vitro* proliferation of mouse uterine cells depends on Ca^{2+} concentration and maximal growth occurs at 0.05 m*M* Ca^{2+}. It has also been reported that FSH regulation of ovarian steroidogenesis is dependent on both Ca^{2+} and calmodulin (7).

It is noteworthy that addition of 0.05 m*M* Ca^{2+} along with 100 m*M* of various nucleotides (the normal concentrations in Ham-F12) blocked the stimulatory effects of FSH on follicular DNA synthesis. It is possible that exogenous nucleotides dilute the pool of labeled thymidine since uridine is readily converted to thymidine by thymidylate synthase.

With the Harlan hamsters, ovine FSH was able to stimulate DNA synthesis in even the smallest preantral follicles (Stages 1–4), consistent with previous observations (1). However, 8 hr was the optimal time of exposure to FSH compared with 2 hr with the Sasco strain. Moreover, DNA values also did not begin to increase for all stages until 8–12 hr. It is evident that at times there are considerable variations in DNA synthesis by control, baseline incubations. These differences are inexplicable but they point out the necessity of including controls in every experiment with this design. The lack of a dose-dependent increase in DNA synthesis could be due to the fact that only a relatively small population of follicular cells are poised at the G1 phase of the cell cycle and consequently they respond in an all or none fashion. One-hundred nanograms of FSH, the dose used in the definitive experiments, contains approximately 4 ng of LH as a contaminant and 5 ng of LH was only of limited effectiveness in stimulating follicular DNA synthesis. In fact, when given concurrently, 5 ng of LH was antagonistic to the actions of 100 ng of FSH. We cannot offer any explanation for this antagonistic interaction which has been observed previously (1). Stage 1–4 follicles have receptors for FSH and prolactin but not for LH; the latter first appear when thecal cells begin to develop and later spread to granulosa cells in antral follicles (8). The use of recombinant bovine FSH, which is devoid of LH activity, demonstrates that FSH per se can initiate DNA synthesis in some of the larger stages of follicles but not as well as ovine FSH which does have LH contamination. It is possible that a larger dose of recombinant FSH might have stimulated [^{3}H]TdR incorporation in all stages.

Although FSH was the primary stimulus for increasing DNA synthesis, with increasing time (e.g., 12 hr) even control incubations showed dramatic increases in DNA replication. The simple KRBH medium has no added growth factors and it therefore appears that the positive response results from elimination of some constraining factors that are operative *in vivo*. Alternatively, the increase in DNA synthesis could be due directly to intrafollicular growth factor(s) which exert a steady, positive influence on DNA replication. Proestrous hamster follicles are exposed to a tonic, low level of FSH and the latter influences follicular epidermal growth factor content (9).

Based on histologic criteria, follicular development in the hamster was categorized into 10 stages (2). This arbitrary classification, however, is accurately reflected in the amount of DNA/follicle for each stage. For Stages 1 to 5, each successive stage represents an approximate doubling in the amount of DNA per follicle. The largest preantral follicles (Stage 6) initiate a new growth phase culminating with a 10-fold increase in DNA content in large preantral follicles (Stage 10). Thus, the DNA values offer further evidence for the validity of the follicular classification scheme.

It appears that the original Sasco stock of hamsters which was utilized in our previous experiments was much more sensitive and responsive to endogenous and exogenous FSH than the new Harlan strain. With the phasing out of the Sasco breeding colony, however, the Harlan hamsters show enough similarities to serve as a model for factors regulating follicular selection. The experiments point out that important strain differences exist even among hamsters and this should be taken into account in reporting results from other laboratories.

This work was supported by a Grant HD 00596 from NIH. We thank the National Pituitary Program for providing the ovine FSH and LH and Dr. Scott Chappel for the recombinant bovine FSH.

1. Roy SK, Greenwald, GS. *In vitro* effects of follicle stimulating hormone, luteinizing hormone and prolactin in follicular deoxyribonucleic acid synthesis in the hamster. Endocrinology **122**:952–958, 1988.
2. Roy SK, Greenwald GS. An enzymatic method for dissociation of intact follicles from the hamster ovary: histological and quantitative aspects. Biol Reprod **32**:203–215, 1985.
3. Roy SK, Greenwald GS. A quantitative analysis of *in vitro* incorporation of ^{3}H-thymidine into hamster follicles during the estrous cycle. J Reprod Fertil **77**:143–152, 1986.
4. Chappel S, Beck A, Nugent N, Hyman L. Production of bovine follicle stimulating hormone (FSH) by recombinant DNA technology [Abstract 130]. Program Endocrine Society, 69th Annual Meeting, Endocrinology 120 (suppl), 1987.
5. Greenwald GS, Terranova PF. Follicular selection and its control. In: Knobil E, Neill J, Eds. Physiology of Reproduction. New York: Raven Press, pp 387–446, 1988.
6. Tomooka Y, DiAugustine RP, McLachlan JA. Proliferation of mouse uterine epithelial cells *in* vitro. Endocrinology **118**:1011–1018, 1986.
7. Carnegie JA, Tsang BK. Microtubules and the calcium-dependent regulation of rat granulosa cell steroidogenesis. Biol Reprod **36**:1007–1015, 1987.
8. Roy SK, Wang SC, Greenwald GS. Radioreceptor and autoradiographic analyses of FSH, hCG and prolactin binding sites in primary to antral hamster follicles during the periovulatory period. J Reprod Fertil **79**:307–313, 1987.
9. Roy SK, Greenwald GS. Immunocytochemical localization of EGF-like activity in the hamster ovary with a polyclonal antibody. Endocrinology **126**:1309–1317, 1990.