Cyclic changes in messenger RNAs encoding inhibin/activin subunits in the ovary of the golden hamster (Mesocricetus auratus)

Koji Y Arai, Hisashi Kishi1, Satoshi Onodera1,2, Wanzhu Jin1, Gen Watanabe1,3, Akira K Suzuki4, Shinji Takahashi5, Toshihiko Kamada2, Toshio Nishiyama and Kazuyoshi Taya1,3

Department of Tissue Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
1Laboratory of Veterinary Physiology, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
2Laboratory of Animal Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan
3Department of Basic Veterinary Science, The United Graduate School of Veterinary Science, Gifu University, Gifu 501-1193, Japan
4Air Pollutants Health Effect Team, National Institute of Environmental Studies, Ibaraki 305-0053, Japan
5Ecological Effect Research Team, National Institute of Environmental Studies, Ibaraki 305-0053, Japan

(Requests for offprints should be addressed to K Taya; Email: taya@cc.tuat.ac.jp)

Abstract

To elucidate changing patterns of inhibin/activin subunit mRNAs in the ovary of the golden hamster (Mesocricetus auratus) during the oestrous cycle, inhibin/activin subunit cDNAs of this species were cloned and ribonuclease protection assay and in situ hybridization were carried out. Inhibin α-subunit mRNA was localized in granulosa cells of primary, secondary, tertiary and atretic follicles throughout the 4-day oestrous cycle. It was also expressed in luteal cells on days 1 (oestrus), 2 (metoestrus) and 3 (dioestrus). βA-subunit mRNA was localized in granulosa cells of large secondary (>200 µm) and tertiary follicles throughout the oestrous cycle. βB-subunit mRNA was confined to granulosa cells of large secondary and tertiary follicles. Both α- and βA-subunit mRNAs were also found in ovarian interstitial cells and theca interna cells of secondary and tertiary follicles in the ovary of the golden hamster (Mesocricetus auratus) during the preovulatory period. The expression pattern of βA-subunit mRNA during the preovulatory period is unique and not found in other species. An i.v. injection of anti-luteinizing hormone-releasing hormone (LHRH) serum before the LH surge abolished the expression of α- and βA-subunit mRNAs in ovarian interstitial cells and theca interna cells. The treatment also abolished the preovulatory increase in βA-subunit mRNA. Furthermore, administration of human chorionic gonadotrophin (hCG), which followed the injection of anti-LHRH serum, restored the expression patterns of α- and βA-subunit mRNAs. The present study revealed that the golden hamster showed a unique expression pattern of βA-subunit mRNA in response to the LH surge.

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Introduction

The reproductive cycle of the golden hamster (Mesocricetus auratus) has some unique characteristics as compared with the rat and mouse. For example, the corpus luteum in the cyclic ovary always begins to show signs of histological regression by 3 days after ovulation and almost completely vanishes by the next ovulation (Grady & Greenwald 1968). In addition, the follicle-stimulating hormone (FSH) surge during the preovulatory period is clearly separated into two peaks in the golden hamster (Bast & Greenwald 1974, Kishi et al. 1995) unlike the rat in which the FSH surges almost overlap (Watanabe et al. 1990).

Inhibins, which belong to the transforming growth factor-β (TGF-β) superfamily, were initially isolated from ovarian follicular fluid as peptides that preferentially inhibit FSH secretion from the pituitary gland (de Jong 1988, Ying 1988). Two inhibins have been identified. Each inhibin is composed of a common α-subunit and either a βA-subunit to give rise to inhibin A or a βB-subunit to form inhibin B (de Jong 1988, Ying 1988). On the other hand, the βA- and βB-subunits of inhibin form homo- and hetero-dimers termed activin A, activin AB, and activin B (de Jong 1988, Ying 1988). Although activins were initially isolated as gonadal peptides that stimulate FSH secretion (de Jong 1988, Ying 1988), they are now known to be multifunctional growth factors that are very important for vertebrate development and other physiological events (Mather et al. 1992, 1997, Hillier & Miro 1993, Findlay et al. 2001). Inhibins are known to be major

Previous studies have demonstrated that expression of mRNAs encoding inhibin/activin subunits in the ovary shows noticeable changes during the rat oestrous cycle (Meunier et al. 1988, Woodruff et al. 1988, Arai et al. 2002), and the changing patterns of ovarian βA- and βB-subunit mRNAs were very similar to those of plasma inhibin A and inhibin B respectively (Arai et al. 2002). Our previous data demonstrated that changing patterns of plasma inhibin A and inhibin B in the golden hamster were discordant during the oestrous cycle (Ohshima et al. 1999), as observed in the rat (Woodruff et al. 1996, Arai et al. 2002). The changing pattern of plasma inhibin B in the golden hamster was very similar to that in the rat. However, plasma concentrations of inhibin A showed a striking rise during the preovulatory period (Ohshima et al. 1999), which was not observed in rats (Woodruff et al. 1996, Arai et al. 2002), cows (Kaneko et al. 2002) and humans (Muttukrishna et al. 1994). Although these data suggest that the golden hamster ovary would show a unique changing pattern of βA-subunit mRNA during the preovulatory period, cyclic changes in levels and distribution patterns of inhibin/activin subunit mRNAs in the golden hamster ovary remain unknown. To elucidate the changing patterns of inhibin/activin subunit mRNAs in the ovary of this species, we cloned cDNAs encoding α-, βA- and βB-subunits from the golden hamster ovary by rapid amplification of cDNA ends (RACE) and examined expression patterns of these mRNAs by ribonuclease protection assay and in situ hybridization during the oestrous cycle. Furthermore, we examined the effects of the preovulatory luteinizing hormone (LH) surge on inhibin/activin subunit mRNA expression by administering antiserum to LH-releasing hormone (LHRH).

Materials and Methods

Animals

Adult cyclic golden hamsters (Mesocricetus auratus; 2–3 months old) were kept under controlled temperature and lighting (lights on from 0500 to 1900 h) conditions. The 4-day oestrous cycle was monitored by the presence of characteristic vaginal discharge in the morning of the day of ovulation, which was designated day 1 of the oestrous cycle. Golden hamsters with at least two consecutive 4-day oestrous cycles were used in this study. The animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals prepared at Tokyo University of Agriculture and Technology.

Cloning of inhibin/activin subunit cDNAs by RACE

Female golden hamsters were anaesthetized with ether and killed by decapitation. The ovaries were immediately frozen in liquid nitrogen and stored at −80°C until isolation of total RNA with TRIzol reagent (Invitrogen). Ovarian poly A(+) RNA was prepared with Oligotex-dT30 Super (Roche), and then 3′- and 5′-RACE were done as previously described (Arai et al. 2003). An adaptor primer (5′-GACCACCGGCCCTAGTCATAC-3′) and gene-specific primers as listed in Table 1 were used. Gene-specific primers were designed by choosing the homologous DNA sequence regions between rat, mouse and human homologues of inhibin/activin subunit cDNAs, which were available from GenBank (National Centre for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA). The PCR products were subcloned into pGEM-T easy vector (Promega) and subjected to sequence analysis.

RNA probes

Template cDNAs for complementary RNA probes were prepared by RT-PCR using the gene-specific primers listed in Table 1. Template α-subunit cDNA was amplified with α-sense primer and α-antisense primer. Template βA-subunit cDNA for in situ hybridization was amplified with βA-sense primer 1 and βA-antisense primer 1. Template βA-subunit cDNA for ribonuclease protection assay was prepared with βA-sense primer 2 and βA-antisense primer 2. Template βB-subunit cDNA was prepared with βB-sense primer 1 and βB-antisense primer 2.

**Table 1 Gene-specific primers for inhibin/activin subunits**

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>α-sense primer</td>
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<tr>
<td>α-antisense primer</td>
<td>5′-GCTAGGAGAGTTTCTGTTGGCA-3′</td>
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<td>βA-sense primer 1</td>
<td>5′-TCAAGGGAGAGGGATACA-3′</td>
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<tr>
<td>βA-sense primer 2</td>
<td>5′-GTGATGATCTCGAGGTCTGCT-3′</td>
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<tr>
<td>βA-antisense primer 1</td>
<td>5′-CACCTGACAGTTCCGCTGGAG-3′</td>
</tr>
<tr>
<td>βA-antisense primer 2</td>
<td>5′-GACCAGCGGCCCTAGTCATAC-3′</td>
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</table>
primer. Amplified cDNAs were subcloned into pGEM-T easy vector (Promega). The golden hamster GAPDH cDNA clone was obtained by 3’-RACE using the GAPDH-sense primer, 5’-ACCACAGTCCATGCCCAT CAC-3’. The 3’-RACE product was subcloned into pGEM-T easy vector. Thereafter, the GAPDH cDNA was digested with EcoRI, and the 429 bp fragment of golden hamster GAPDH cDNA was subcloned into pBluescript II SK(+) vector (Stragagene, La Jolla, CA, USA) as a template for the RNA probe. DIG-labelled complementary RNA probes were synthesized with SP6, T3 or T7 RNA polymerase using DIG RNA labelling mixture (Roche) according to the manufacturer’s protocols.

Ribonuclease protection assays

Golden hamsters were killed by decapitation at 1100 h on days 1 (oestrus), 2 (metoestrus), 3 (dioestrus) and at 1100 and 1700 h on day 4 (pro-oestrus). Three animals were killed at each point. Total ovarian RNA was isolated as described (Arai et al. 2001, 2002). Ten micrograms total RNA were used for detecting α-subunit mRNA; 20 µg total RNA were used for detecting βA-subunit, βB-subunit and GAPDH mRNAs. The latter two mRNAs were hybridized in a single tube.

Preparation of ovarian sections of cyclic golden hamsters

Animals were killed by decapitation at 1100 h on each day of the oestrous cycle; at 0500, 1700 and 2300 h on day 4; and at 0500 h on day 1. The ovaries were fixed in freshly prepared 4% (w/v) paraformaldehyde (PFA; Sigma) in 0·01 M PBS and embedded in paraffin. The paraffin-embedded ovaries were serially sectioned at 6 µm thickness and placed on glass slides coated with 3-aminopropyltriethoxysilane. The sections were subjected to in situ hybridization for inhibin/activin subunit mRNAs or haematoxylin-eosin staining. Ovaries from at least three animals were examined at each sampling point.

Effects of anti-LHRH serum and human chorionic gonadotrophin (hCG) on inhibin/activin subunit mRNA expression in the ovary

To elucidate the effects of preovulatory LH surge on expression of ovarian inhibin/activin subunit mRNAs, animals received an i.v. injection of 200 µl anti-LHRH serum at 1100 h on day 4 followed by an i.v. injection of either 10 IU hCG (Sankyo Zoki Ltd, Tokyo, Japan) or saline at 1300 h. This administration protocol of the antiserum was shown to block the occurrence of the LH surge (Kishi et al. 2002). At 1700 h on day 4, animals were killed by decapitation and ovaries were collected. Ovaries were subjected to isolation of total RNA or preparation of ovarian sections as described above.

In situ hybridization

The sections were deparaffined with xylene, and rehydrated with a graded series of ethanol, and washed with 0·01 M PBS for 5 min twice. Thereafter, the sections were treated with 0·2 M HCl for 15 min at 37°C, digested with 20 µg/ml proteinase K in 0·01 M PBS for 15 min at 37°C, fixed in 4% PFA in 0·01 M PBS for 30 min, and treated with 0·2% glycine in 0·01 M PBS for 5 min. The sections were washed with 0·01 M PBS between treatments. Subsequently, the sections were prehybridized in hybridization buffer (50% deionized formamide, 0·3 M NaCl, 20 mM Tris–HCl (pH 8·0), 1 × Denhardt’s solution, 0·5 mg/ml Escherichia coli tRNA) for 30 min at 37°C. Complementary RNA probes diluted in hybridization buffer (1–2 µg/ml) were denatured for 5 min at 90°C and hybridized overnight at 50°C under coverslips in a humidified box. After hybridization, the sections were washed in 2 × SSC/50% formamide for 30 min at 50°C, treated with RNase solution (10 mM Tris–HCl (pH 8·0), 500 mM NaCl, 20 µg/ml RNaseA) for 15 min at 37°C, and washed again with 1 × SSC/50% formamide for 15 min at 50°C. Before and after the treatment with RNaseA, the sections were washed with RNase diluent for 5 min twice at 37°C. Thereafter, the sections were washed in buffer 1 (0·1 M Tris–HCl, 0·15 M NaCl, pH 7·5) for 5 min, incubated with buffer 2 (buffer 1 containing 1% blocking reagent (Roche)) for 20 min at 37°C, followed by alkaline phosphatase-conjugated anti-DIG immunoglobulin G (IgG; Roche) diluted with buffer 2 (1:500) for 1 h at 37°C. After washing with buffer 1 for 5 min and buffer 3 (0·1 M Tris–HCl, 0·1 M NaCl, 0·05 M MgCl2, pH 9·5) for 10 min at room temperature, cRNA probes were visualized using nitroblue tetrazolium salt (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche). The reaction was stopped in water, and the sections were mounted. For negative controls, sense strand RNA probes were used instead of complementary RNA probes.

Statistical analysis

Values are presented as means ± s.e.m. To compare the mean values, results were subjected to an ANOVA, followed by a Student–Neuman–Keuls test. Differences were considered significant at P < 0·05.

Results

Cloning of complementary DNAs encoding golden hamster inhibin/activin subunits and their deduced amino-acid sequences

The 5′- and 3′-RACE products for α-subunit revealed a 1·4 kb cDNA. The α-subunit cDNA contained a
complete open reading frame, which consists of 1101 nucleotides. Unfortunately, we could not obtain 5'-RACE products for either α-subunit. However, 3'-RACE and usual PCR products revealed a 1.3 kb cDNA for α-subunit that contained 1239 base protein coding region, and a 2.8 kb cDNA for β-subunit that contained 987 base protein coding region. A poly adenylation signal (AATAAA) was found in α-subunit cDNA at 148 bases downstream from the stop codon, and in β-subunit cDNA at 1789 bases downstream from the stop codon. However, consensus polyadenylation signal was not found in β-subunit cDNA, suggesting that reverse transcription started from a short polyadenine sequence in the 3'-untranslated region of α-subunit mRNA. Another possibility is that the β-subunit mRNA has an atypical polyadenylation signal. The nucleic acid sequences of golden hamster inhibin/activin subunits are available from the DNA Data Base of Japan (DDBJ; Mishima, Shizuoka, Japan). The accession numbers are AB187511, AB187512 and AB187513 for α-, α- and β-subunit cDNAs respectively. The deduced amino-acid sequences of golden hamster inhibin/activin subunits were determined according to the protein sequences of inhibin/activin subunits of other species. Identical amino-acid sequences are indicated by dots. Missing amino-acid residues are indicated by dashes.

Figure 1 A comparison of the deduced amino-acid sequence of golden hamster (g. hamster) inhibin α-subunit precursor with other species. Gene bank accession numbers of the protein sequences of rat, mouse, Siberian hamster (Phodopus sungorus, S. hamster), human, pig (porcine), cow (bovine) and horse (equine) are AAA41435, AAA39314, AF432351, 1608260A, WFPGA, P07994 and BAA08863 respectively. The first amino acid of the mature protein was numbered +1. In Figs 1 to 3, the positions of the first amino acids of mature golden hamster inhibin/activin subunits were determined according to the protein sequences of inhibin/activin subunits of other species. Identical amino-acid sequences are indicated by dots. Missing amino-acid residues are indicated by dashes.
The amino-acid sequence of the predicted mature form of the golden hamster inhibin βα-subunit shares 94·7, 95·5, 95·5, 80·6, 85·1, 87·3 and 85·8 homology with those of rat, mouse, Siberian hamster, human, pig, cow and horse respectively. The golden hamster α-subunit cDNA revealed the partial amino-acid sequence of the α-subunit, which corresponded to the C-terminus 412-amino-acid residues (Fig. 2). The partial protein sequence of the α-subunit shares 99·0, 98·5, 95·4, 90·5, 94·4 and 91·1% homology with those of rat, mouse, human, pig, cow and horse respectively. The amino-acid sequence of the predicted mature form of the golden hamster ββ-subunit shares 100% homology with those of rat, mouse, human, pig and cow, and 97·4% homology with that of the horse. The golden hamster βα-subunit cDNA revealed the partial amino-acid sequence of the βα-subunit, which corresponded to C-terminus 328-amino-acid residues (Fig. 3). The partial protein sequence of golden hamster ββ-subunit shares 99·1, 99·1, 96·3, 95·7 and 95·1% homology with those of rat (B41398), mouse (P09529), human (AAA59451), pig (AAM66766) and cow (P42917) respectively. The amino-acid sequence of the predicted mature form of the golden hamster ββ-subunit shares 100, 100, 96·5, 96·5 and 97·4% homology with those of rat, mouse, human, pig and cow respectively.

Changes in inhibin/activin subunit mRNA levels in the golden hamster ovary during the oestrous cycle

Levels of three inhibin/activin subunit mRNAs showed different changing patterns during the oestrous cycle (Fig. 4). Levels of α-subunit mRNA in the ovary continuously increased from day 2 to the evening of day 4 and returned to the basal levels by 1100 h on day 1. Levels of...
Aβ-subunit mRNA remained low from day 1 to the morning of day 4. At 1700 h on day 4, levels of Aβ-subunit mRNA showed a remarkable increase. Levels of Bβ-subunit mRNA remained at relatively high levels from day 2 to the morning of day 4, considerably decreased in the evening of day 4, and recovered by the morning of day 5. The changing patterns of plasma inhibins during the 4-day oestrous cycle of the golden hamster in our colony, which have been reported previously (Ohshima et al. 1999), are shown in Fig. 5 for comparison. The changing patterns of α-, βA- and βB-subunit mRNAs are similar to those of plasma inhibin pro-αC, inhibin A and inhibin B respectively.

Changes in localization of inhibin/activin subunit mRNAs in the golden hamster ovary during the oestrous cycle

The following terminology was used to describe the stages of follicle maturation: primary follicle, an oocyte was surrounded by one to several layers of granulosa cells (up to 150 µm); secondary follicle, the maturing oocyte increases in size and becomes surrounded by more granulosa cell layers (150–250 µm); tertiary follicle, this follicle is characterized by a fluid-filled antrum (>250 µm). Some of the large tertiary follicles (up to 600 µm) will be selected for ovulation in the early morning of day 1 while others will undergo atretic processes. Preovulatory follicles can be distinguished from non-ovulatory large tertiary follicles on a histological basis, in which case changes in various cells types and structures surrounding the basal lamina can be observed.

Representative photographs of in situ hybridization are shown in Fig. 6. Strong signals for α-subunit mRNA were detected in granulosa cells of large secondary (>200 µm) and tertiary follicles throughout the oestrous cycle (Fig. 6). α-Subunit mRNA was also found in granulosa cells of primary and small secondary (<200 µm) follicles on each day of the oestrous cycle, and in luteal cells on days 1, 2 (Fig. 6i) and 3. However, the signals were weak as

Figure 3 A comparison of the deduced amino-acid sequence of golden hamster inhibin βB-subunit with those of other mammalian species. Gene bank accession numbers of the protein sequences of rat, mouse, human, pig and cow are B41398, P09529, AAA59451, AAM66766 and P42917 respectively. See Fig. 1 for details.
compared with large secondary and tertiary follicles. In the evening of day 4, signals for α-subunit mRNA were also detected in theca interna cells of tertiary and atretic follicles and in ovarian interstitial cells (Fig. 6f and k). Moderate signals for α-subunit mRNA were detected in granulosa cells of atretic follicles throughout the oestrous cycle, while signals for βA- and βB-subunit mRNAs were absent. The intensity of the signals for α-subunit mRNA in atretic follicles was negatively correlated with the progress of atresia. βA-subunit mRNA was found in granulosa cells of large secondary and tertiary follicles throughout the oestrous cycle. It was also expressed in some small secondary follicles. Interestingly, βA-subunit mRNA was found in ovarian interstitial cells and in theca interna cells of tertiary and atretic follicles in the evening of day 4 (Fig. 6f and l). This distribution pattern of βA-subunit mRNA was not found in other species. βB-subunit mRNA was localized in granulosa cells of large secondary, tertiary and some small secondary follicles throughout the oestrous cycle. Expression of βB-subunit mRNA in tertiary follicles decreased from the morning of day 3 to the morning of day 4 (Fig. 6d and e). The decrease in βB-subunit mRNA in tertiary follicles started from peripheral granulosa cells. Expression of βB-subunit mRNA in tertiary follicles was very low in the evening of day 4, while the strongest expression of α- and βA-subunit

Figure 4 (a) Changes in inhibin/activin subunit mRNA levels in the golden hamster ovary during the oestrous cycle: mRNAs levels were examined by ribonuclease protection assays. The signals were analyzed with NIH image and results are expressed as intensity relative to GAPDH mRNA. Values are means ± S.E.M. for three independent experiments. Bars with the same marks represent the minimum difference between values required for the points to be significantly different (P<0.05). (b) Representative images of ribonuclease protection assays detecting α-, βA- and βB-subunit mRNAs and GAPDH mRNA.
mRNAs was observed in tertiary follicles at this time (Fig. 6f). At 2300 h on day 4, only α-subunit mRNA was moderately expressed in preovulatory follicles (Fig. 6g). The relative abundance of inhibin/activin subunit mRNAs in granulosa cells of various follicles is summarized in Table 2.

**Effects of anti-LHRH serum on levels of ovarian inhibin/activin subunit mRNAs during the preovulatory period**

The effects of anti-LHRH serum on ovarian inhibin/activin subunit mRNA levels are shown in Fig. 7. RNA samples of intact animals at 1100 and 1700 h on day 4 were also examined for comparison. Administration of the anti-LHRH serum completely blocked the preovulatory rise of βA- and the preovulatory decrease in βB-subunit mRNA levels (Fig. 7). Furthermore, an injection of hCG after the administration of the anti-LHRH serum partially restored the preovulatory increase in βA-subunit mRNA. In addition, the hCG treatment could decrease βB-subunit mRNA levels. These results strongly indicate that the preovulatory LH surge induces the striking rise in βA-subunit mRNA and the noticeable decrease in βB-subunit mRNA at 1700 h on day 4. On the other hand, the administration of anti-LHRH serum tended to decrease α-subunit mRNA levels, and the hCG treatment moderately increased α-subunit mRNA.

**Effects of anti-LHRH serum on localization of ovarian inhibin/activin subunit mRNAs during the preovulatory period**

Administration of anti-LHRH serum at 1100 h on day 4 abolished the expression of α- and βA-subunit mRNAs in theca interna and ovarian interstitial cells at 1700 h on day 4 (Fig. 8a). In contrast, an injection of hCG, which followed the administration of anti-LHRH serum, restored α- and βA-subunit mRNA expression in theca interna and ovarian interstitial cells (Fig. 8c and e). With respect to βB-subunit mRNA, it was still detected in tertiary follicles of the animals treated with anti-LHRH serum alone (Fig. 8a), while it was abolished by an injection of hCG that followed the treatment with anti-LHRH serum (Fig. 8c).

**Discussion**

The present study is the first to demonstrate deduced amino-acid sequences of golden hamster inhibin/activin subunits and changing patterns of inhibin/activin subunit mRNAs in the golden hamster ovary during the oestrous cycle.

The primary structures of golden hamster inhibin/activin subunits confirmed that amino-acid sequences of...
α-subunits are rather diverse between species, as compared with those of β-subunits. In particular, mature forms of golden hamster, rat, mouse, human, porcine and bovine βA-subunits show 100% identity, indicating that conservation of the amino-acid sequence of the βA-subunit is very important for its function. Northern-blot hybridization using ovarian total RNA detected: 1·5 and 3·4 kb α transcripts; 1·3, 1·5 and 6·2 kb βA transcripts; and 1·5 and 3·4 kb βB transcripts (data not shown). The variation in size of inhibin/activin subunit mRNAs has been shown in other species (Mason et al. 1985, Esch et al. 1987, Woodruff et al. 1987, Chen & Johnson 1996, Yamanouchi et al. 1997). According to the sizes of the PCR products, α- and βB-subunit cDNAs that we have obtained are probably 1·5 and 3·4 kb in length respectively. The full size of the βA-subunit cDNA that we have cloned is not known because we did not find the consensus polyadenylation signal in the βA-subunit cDNA.

The distribution pattern of α-subunit mRNA in the golden hamster ovary is consistent with previous observations in the rat (Meunier et al. 1988). Because expression of α-subunit mRNA in granulosa cells increased as follicles developed, the increase in ovarian α-subunit mRNA levels during the follicular phase is probably due to development of large follicles to be selected for ovulation. However, previous studies indicated that in the golden hamster, the ovary of day 3 was most abundant in healthy large follicles, and the number of healthy large follicles decreased from day 3 to the morning of day 4 (Greenwald 1991, Kishi et al. 1995). Therefore, α-subunit mRNA levels do not reflect merely the number of large healthy follicles. Because considerable levels of α-subunit mRNA were detected in early atretic follicles, it may be responsible for the discordance between α-subunit mRNA levels and the number of large healthy follicles. α-Subunit mRNA was also found in ovarian interstitial cells and theca interna cells during the preovulatory period, as observed in the rat ovary (Meunier et al. 1988, Rivier et al. 1989). Involvement of the LH surge in the regulation of thecal and interstitial α-subunit mRNA expression is also consistent with the results in the rat (Rivier et al. 1989). Expression of α-subunit mRNA in these types of cells may partly contribute to the increase in α-subunit mRNA in the evening of day 4.

The expression pattern of βA-subunit mRNA in granulosa cells of the golden hamster ovary is consistent with a previous study in the rat (Meunier et al. 1988). However, in the golden hamster, βA-subunit mRNA was also expressed in ovarian interstitial cells and theca interna cells in response to the LH surge or administration of hCG. This distribution pattern of βA-subunit mRNA has not been observed in the rat or in other species.

### Table 2 Relative abundance of inhibin/activin subunit mRNAs in granulosa cells of various follicles in the golden hamster ovary during the oestrous cycle. Sections from at least three animals were examined at each sampling point.

<table>
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<tr>
<th>Time during oestrous cycle</th>
<th>Day 1 (0500 h)</th>
<th>Day 1 (1100 h)</th>
<th>Day 2 (1100 h)</th>
<th>Day 3 (1100 h)</th>
<th>Day 4 (1700 h)</th>
<th>Day 4 (2300 h)</th>
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<td>Atretic follicle</td>
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The following rating was observed: –, no signal; +, signal is faint but distinguishable from background; ++, signal is solid and easy to distinguish from background; ++++, signal is very strong (this rating is only applied for α and βA mRNAs in granulosa cells of tertiary follicles at 1700 h on day 4).
Furthermore, the golden hamster ovary expressed noticeably high levels of βA-subunit mRNA during the preovulatory period, which was not observed in the rat (Arai et al. 2002). A previous study has shown that there are LH/hCG binding sites in the interstitial tissue of the golden hamster ovary (Oxberry & Greenwald 1982), indicating that LH directly affects ovarian interstitial cells to induce expression of α- and βA-subunit mRNAs. Since the strongest signals for βA-subunit mRNA during the oestrous cycle were found in granulosa cells of tertiary follicles at this time, the increase in βA-subunit expression in large follicles was probably a cause of the abrupt increase in βA-subunit mRNA levels. Furthermore, the expression of βA-subunit mRNA in ovarian interstitial cells may also contribute to the increase in βA-subunit mRNA levels since the interstitial tissue occupies a large area in the ovary. It is likely that the preovulatory rise in βA-subunit mRNA preferentially increased activin A secretion because

![Figure 7](image)

**Figure 7** (a) Effects of anti-LHRH serum on levels of ovarian inhibin/activin subunit mRNAs during the preovulatory period. Animals were given an i.v. injection of 200 μl anti-LHRH serum at 1100 h on day 4. Thereafter, the animals received an i.v. injection of either saline (LHRH-AS) or 10 IU hCG (LHRH-AS+hCG) at 1300 h. They were killed at 1700 h on day 4 and ovarian total RNA samples were collected. mRNA levels were examined by ribonuclease protection assays. Samples obtained from intact animals at 1100 and 1700 h on day 4 were also examined for comparison. The signals were analyzed with NIH image and results are expressed as intensity relative to GAPDH mRNA. Values are means ± S.E.M. for three independent experiments. Bars with the same marks represent the minimum difference between values required for the points to be significantly different (P<0.05). (b) Representative images of ribonuclease protection assays detecting α-, βA- and βB-subunit mRNAs and GAPDH mRNA.
α-subunit mRNA did not show noticeable increase as compared with βA-subunit mRNA. The increase in βA-subunit mRNA may have some roles in the regulation of follicular development because activin affects functions of ovarian cells (Hillier & Miro 1993, Findlay et al. 2001).

In addition, the increase in βA-subunit mRNA may be responsible for the low levels of plasma progesterone in the golden hamster (Kishi et al. 1995) as compared with the rat (Watanabe et al. 1991) because activin is known to suppress progesterone secretion from both granulosa...
(Shukovski & Findlay 1990, Li et al. 1992) and luteal cells (Branian et al. 1992, Di Simone et al. 1994).

Throughout the oestrous cycle, the changing pattern of ββ-subunit mRNA in the golden hamster ovary is consistent with that in the rat (Meunier et al. 1988, Arai et al. 2002). As observed in the rat (Meunier et al. 1988), ββ-subunit mRNA in tertiary follicles decreased as maturation of follicles advanced, whereas that of βα-subunit mRNA remained high until the evening of day 4. The decrease in ββ-subunit mRNA in tertiary follicles started from peripheral granulosa cells, indicating that there are physiological differences between inner and outer layer of granulosa cells in large tertiary follicles. Results of both in situ hybridization and ribonuclease protection assay revealed a noticeable decrease in ovarian ββ-subunit mRNA levels in the evening of day 4 and the experiments using anti-LHRH serum clearly indicate that the LH surge induced the preovulatory decrease in ββ-subunit mRNA. This abrupt decrease in ββ-subunit mRNA may be important for triggering the secondary FSH surge and subsequent recruitment of developing follicles.

The changing patterns of ovarian βα- and ββ-subunit mRNAs in this study were very similar to those of circulating inhibin A and inhibin B (Ohshima et al. 1999) respectively. In particular, the abrupt increase in βα-subunit mRNA is in agreement with the noticeable increase in plasma concentrations of inhibin A in the evening of day 4 that is characteristic in the golden hamster. The preovulatory increase in inhibin A may be responsible for the clear separation of the primary and secondary FSH surges in this species (Bast & Greenwald 1974, Kishi et al. 1995).

Previous studies have shown that both inhibin and activin affect steroidogenesis of theca cells (Hillier et al. 1991a, 1991b), suggesting that inhibin A and activin A of theca interna origin may regulate steroid secretion by theca cells via autocrine or paracrine fashion during preovulatory period, i.e., the LH surge may modulate steroid secretion by theca cells through induction of inhibin A and activin A secretion in addition to the direct effects of LH.

In the present study, α-subunit mRNA was found in corpora lutea while βα- and ββ-subunit mRNAs had already disappeared from preovulatory follicles at 2300 h on day 4. Similarly, follicles, which undergo atresia, expressed only α-subunit mRNA. These results suggest that the transcription of α-subunit mRNA is sustained longer than that of βα- and ββ-subunit mRNAs during differentiation from granulosa cells to luteal cells or during atretic processes. Because either βα- or ββ-subunit is required to secrete biologically active dimers, strict regulation of α-subunit mRNA may not necessary. It is also possible that the α-subunit monomer has some physiological roles through unknown biological activities.

In summary, the present study revealed the primary structures of inhibin/activin subunits of the golden hamster, the unique expression pattern of βα-subunit mRNA in the golden hamster ovary and its induction by the preovulatory LH surge. The unique expression pattern of βα-subunit mRNA may be a cause of some of the unique reproductive features of the female golden hamster.

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