Fetal expression of GnRH and GnRH receptor genes in rat testis and ovary

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Abstract

The identification of gonadal gonadotropin-releasing hormone receptors (GnRH-R) and evidence of direct inhibitory effects of GnRH agonists upon steroidogenesis in adult rat gonads, lend credence to a putative intragonadal role of a locally secreted GnRH or GnRH-like peptide. Using reverse transcription-polymerase chain reaction followed by Southern blot hybridization and sequencing, we identified, both in the ovary and in the testis of fetal and adult rats, a fully processed GnRH messenger RNA (mRNA), the sequence of which, in adult testis, was identical to that found in the hypothalamus. We also detected in the testis, but not in the ovary, a transcript containing the first intron.

The ontogeny of GnRH and GnRH-R gene expression was studied in rat gonads from 14.5 to 21.5 days post-coitum (dpc), using dot blot hybridization of total RNA. During this period, the levels of cyclophilin mRNA

normalized to total RNA remained unchanged. Thus, we used cyclophilin as an internal standard. GnRH mRNA was detected in the ovary at 18.5 dpc, four days later than in the testis, and similar levels were found in both sexes at birth. GnRH-R mRNA was present at 14.5 dpc in the testis and at 15.5 dpc in the ovary, with the levels at 21.5 dpc being 2.4 times higher in the testis than in the ovary. GnRH and GnRH-R mRNA levels increased in both sexes in late fetal development, but this increase appeared two days sooner in the ovary compared with the testis, thus supporting the hypothesis that expression of the GnRH and GnRH-R genes is regulated in a sex-dependent manner during fetal development. In all cases, expression of GnRH and GnRH-R preceded gonadotropin receptors in the gonads and initiation of gonadotropin secretion by the pituitary.

Journal of Endocrinology (1998) **159**, 179–189

Introduction

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that plays a key role in the neuro-hormonal control of reproduction by stimulating the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), via specific membrane receptors (GnRH-R) of the gonadotrope cells. These gonadotropins promote, in turn, the development of gonadal functions such as gametogenesis and steroidogenesis.

There is strong evidence for the presence of specific GnRH or GnRH-like binding sites on the gonads of the adult rat (Hsueh & Jones 1981). Radioligand binding assays first demonstrated their existence on Leydig cells (Clayton *et al.* 1980, Lefebvre *et al.* 1980, Sharpe & Fraser 1980a) and on granulosa and luteal cells (Clayton *et al.* 1979, Harwood *et al.* 1980a,b, Reeves *et al.* 1980). We have shown that the primary structure of this gonadal

GnRH-R is identical to the pituitary one (Moumni *et al.* 1994). Since the hypothalamic GnRH cannot be the ligand of this gonadal GnRH-R, because of its low concentration in the general circulation (Nett *et al.* 1974) and of its short half-life (Eskay *et al.* 1977, Hsueh & Jones 1981), the existence of an endogenous GnRH-like peptide was suspected. It was demonstrated in Sertoli cells by competition studies and immunochemistry (Sharpe & Fraser 1980b, Paull *et al.* 1981, Bhasin *et al.* 1983), while a peptide with binding activity similar to GnRH was described in granulosa cells (Aten *et al.* 1986). However, to date the exact nature of this peptide and its role in gonadal paracrine and autocrine regulation remain uncertain.

In vivo studies in male or female adult hypophysectomized rats have shown that exogenous GnRH or GnRH agonists can both stimulate and inhibit gonadal functions, especially steroidogenesis (Hsueh & Jones 1981). In males, for example, when a GnRH agonist is administered at a

low dose or in a short-term study, testosterone secretion is stimulated (Sharpe *et al.* 1982), but it is inhibited when the agonist is administered at a high dose or in a long-term study (Arimura *et al.* 1979, Hsueh & Erickson 1979). The time-dependence of the GnRH agonist response has been studied in organotypic cultures of fetal and neonatal rat testes (Habert *et al.* 1991). During fetal development, the cellular mechanisms involved in the inhibitory response to GnRH are differentiated 3–5 days before those involved in the stimulating response. These studies suggest that the GnRH-R gene is expressed in the testis early during fetal development, thus raising the question of a potential role of testicular GnRH in triggering gonadal steroidogenesis regulation. In contrast to the testis, little is known about the setting of cellular mechanisms controlling steroidogenesis in the fetal ovary, because the ovary is steroidogenically quiescent until the first postnatal week (for review see Huhtaniemi 1995).

Elucidation of the potential role of gonadal GnRH in the local control of fetal steroidogenesis necessitates the investigation of the expression of the GnRH and the GnRH-R genes in the gonads during development. Therefore, we searched for the presence of GnRH and GnRH-R messenger RNA (mRNA) in rat fetal gonads, using reverse transcription-polymerase chain reaction (RT-PCR) followed by Southern blotting of the PCR products. Furthermore, the sequence of GnRH mRNA in adult rat testis was determined in order to elucidate the nature of the testicular GnRH-like peptide. Finally, gonadal GnRH and GnRH-R mRNA levels were evaluated during fetal development by dot blot hybridization.

Materials and Methods

Animals and tissues

Female and male rats (Sprague–Dawley, Centre d'Élevage Janvier, Le Genest, France) were housed under controlled photoperiods (12 h light:12 h darkness), with water and food available *ad libitum*. Males were caged with the females for one night. Since the estimated time of ovulation was 0200 h, the day following an overnight mating was counted as day 0.5. Pregnant rats were killed at the same hour 14.5 to 21.5 days post-coitum (dpc), and fetuses were removed. The sex of the fetuses was determined by morphological differences and the gonads were dissected together with adjacent mesonephros for 14.5 and 15.5 dpc fetuses, or without mesonephros for 16.5 to 21.5 dpc fetuses. In addition, the stomach and the limb buds were taken as negative controls for dot blot hybridization. The ovaries were not taken from 14.5 dpc fetuses because they were difficult to excise without contamination by other tissues. After a brief rinse in normal saline solution, the tissues were rapidly frozen in liquid nitrogen and stored at -80°C until use.

Postnatal testes were also removed from 4 day post-partum (dpp), 20 dpp and adult (5 months) rats, frozen in liquid nitrogen and stored at -80°C .

RNA extraction

Gonads from the same stage of development were pooled to obtain at least 60 μg RNA for both dot blot and RT-PCR studies. Total RNA was extracted using the Tri-Insta-Pure procedure (Eurogentec, Seraing, Belgium) (Chomczynski & Sacchi 1987).

Reverse transcription and polymerase chain reaction

Total RNA was reverse transcribed to complementary DNA (cDNA) using oligo-dT primers and Superscript II reverse transcriptase (Gibco-BRL, Paris, France), and PCRs were performed with 1 U Taq DNA polymerase (Eurobio, Les Ulis, France) under conditions previously described (Moumni *et al.* 1994). The sets of forward (F) and reverse (R) primers (Genset, Paris, France) used are presented in Fig. 1. F1-R1 was specific for the rat hypothalamic GnRH cDNA (Adelman *et al.* 1986) (Fig. 1A), and F2-R2 (Moumni *et al.* 1994) was specific for the rat pituitary GnRH-R cDNA (Fig. 1B). Amplifications were carried out in a programmable thermal cycler (MJ Research Inc., Watertown, MA, USA) using two different programs of 35 cycles, optimized for GnRH cDNA (50 s at 95°C , 50 s at 50°C , 40 s at 73°C), and GnRH-R cDNA (1 min at 95°C , 1 min at 55°C , 1 min at 72°C). Both programs were terminated by an extension of 10 min at 72°C . For each run of RT-PCR, two negative controls were systematically added, in which water replaced RNA during the RT preparation, or cDNA during the PCR preparation.

Probes and labeling

cDNA probes were used for dot blot hybridization. The GnRH-R probe was described by Moumni *et al.* (1994) and the cyclophilin probe was a gift from Dr J Douglass (Danielson *et al.* 1988). The GnRH probe was a 344 bp cDNA encompassing the entire coding sequence of the GnRH precursor and 68 nucleotides of its 3'-untranslated region (Adelman *et al.* 1986). Probes were labeled by random priming with [α - ^{32}P]dCTP (specific activity 3000 Ci/mmol) (NEN-Dupont de Nemours, Les Ulis, France) using an Amersham Megaprime labeling kit (Amersham, Arlington Heights, IL, USA).

Oligonucleotide probes (Genset) were used for Southern blot hybridization. The sequences and positions of the probes were selected according to the rat GnRH gene sequence (Bond *et al.* 1989) and are indicated on Fig. 1A: the exonic probe E was selected in the second exon, and the intronic probes i1, i2, i3 were selected in the first, second and third intron respectively. A probe J was

Figure 1 Schematic representation of the rat hypothalamic GnRH (A) and pituitary GnRH-R (B) genes. Exons are numbered I to IV for GnRH and I, II, III for GnRH-R, and the coding regions are depicted as filled rectangles. Introns are represented by triangles. Location and orientation of forward (F) and reverse (R) PCR primers are depicted as arrows. Oligonucleotidic exonic (E, J) and intronic (i1, i2, i3) probes used for Southern blot hybridization are represented by heavy dashes. ATG: start codon of translation; TAA: stop codon of translation. (A) GnRH mRNA codes for a 92 amino acid prohormone, consisting of a signal peptide (SP), GnRH, post-translational maturation site (M), and GAP (GnRH-associated peptide). The lengths of introns 1, 2, 3 are 800 bp, 1590 bp and 1350 bp respectively (Bond *et al.* 1989). The expected size of the GnRH PCR product using F1-R1 is 477 bp. (B) The length of intron 1 is at least 12 kb and that of intron 2 approximately 2.5 kb (Reinhart *et al.* 1997). The expected size of the GnRH-R PCR product using F2-R2 is 1038 bp (Moumni *et al.* 1994).

selected in the first exon of the GnRH-R gene (Reinhart *et al.* 1997) as indicated in Fig. 1B. These probes were labeled with [γ - 32 P]ATP (specific activity 3000 Ci/mmol) and T4 polynucleotide kinase (Promega Biotec, Charbonnières, France).

Southern analysis of PCR products

Amplified DNA was electrophoresed on 1.2% agarose gels in Tris-borate-EDTA buffer together with a DNA marker (PhiX-174/HaeIII digested), in the presence of ethidium bromide. DNA was then alkaline-transferred onto nylon filters (Hybond-N, Amersham) and the filters

were hybridized (Sambrook *et al.* 1989). Labeled hybrids were revealed by autoradiography using Kodak AR X-OMAT films (Eastman Kodak, Rochester, NY, USA).

Cloning and sequencing of PCR products

PCR products were purified from agarose using a Qiaex DNA Gel Extraction kit (Qiagen, Courtabœuf, France) and cloned into pUC18 vectors (Surclone Ligation kit, Pharmacia Biotech, Courtabœuf, France) as previously described (Moumni *et al.* 1994). Recombinant plasmid DNA was sequenced bidirectionally using the Sequenase sequencing system version 2.0 (US Biochemicals/

Table 1 Characteristics of the fetal gonad collection

Stage of development (dpc)	Number of litters/total number of fetuses	Number of testes	Number of ovaries	Total RNA extracted/testis (µg)	Total RNA extracted/ovary (µg)
14·5	8/81	74	—	1·2	—
15·5	9/111	110	99	1·5	0·8
16·5	6/60	49	58	1·3	1·3
17·5	6/77	73	77	2·5	1·1
18·5	5/55	63	42	1·9	1·5
19·5	4/43	41	42	3·9	1·8
20·5	2/31	30	32	2·9	2·6
21·5	2/23	16	29	5·9	2·7

Amersham), and M13 universal and reverse primers. Sequence analysis was performed using DNA Strider (Marck 1988).

Dot blot hybridization of fetal tissues RNA

Total RNA from pools of fetal organs of the same stage of development was formaldehyde denatured and diluted aliquots were spotted onto two Protran filters (Schleicher and Schuell, Ecquevilly, France) using a Bio-Rad apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Filter 1 (µg total RNA, 5, 10 and 15 µg aliquots) was hybridized with the [³²P]-labeled GnRH cDNA. Filter 2 (2·5, 5 and 10 µg aliquots) was hybridized with the [³²P]-labeled GnRH-R cDNA. Then both filters were stripped and rehybridized with freshly labeled GnRH-R (filter 1) and GnRH (filter 2) cDNA probes. Finally, both filters were stripped and rehybridized with the [³²P]-labeled cyclophilin probe. Stripping efficiency was systematically controlled by autoradiography. Hybridization and washings were performed using standard methods as previously described (Garrel *et al.* 1993). Radioactivity on the filters was revealed by autoradiography (Kodak X-Omat AR) and quantified by densitometry of the entire surface of the spot using an Autorad 210 device (IMSTAR, Paris, France).

Data analysis

The densitometric data obtained for cyclophilin mRNA were normalized against the total RNA loaded. The results were presented after fitting to the most appropriate model of regression. The significance of the determination coefficient (R²) was assessed using Fisher–Snedecor test, and that of the slope (a) using Student’s *t*-test. A value of *P* ≤ 0·05 was considered significant. The data for GnRH

and GnRH-R mRNA were corrected for variability in loading by expressing the values in arbitrary units as a ratio to cyclophilin.

Results

Gonads collection and RNA extraction

For the study, a total of 481 fetuses from 42 litters was used. The precise number of fetal gonads at each stage of development and the estimated amount of total RNA extracted per gonad, are indicated in Table 1. During the last three days of gestation the amount of total RNA extracted per testis was higher than that extracted per ovary (1·9 to 5·9 µg vs 1·5 to 2·7 µg respectively), whereas it was equal in both sexes until 18·5 dpc.

PCR analysis of GnRH mRNA in adult, immature and fetal gonads

After reverse transcription of total RNA extracted from 14·5 to 21·5 dpc testes, from 15·5 to 21·5 dpc ovaries, and from 4 dpp, 20 dpp and adult testes, parallel amplifications of cDNA were performed using F1–R1. Gel electrophoresis of the products are shown in Fig. 2. The lack of signal in the control RT and PCR reactions omitting template, demonstrated the absence of DNA contaminants and the specificity of the PCR reactions. Two major fragments with sizes of approximately 1280 and 480 bp, were found for 4 dpp (P4), 20 dpp (P20) and adult (Ad) testes (panel A–I). Both hybridized to the exonic [³²P]-labeled probe E (panel A–II). The smallest form was present in larger amounts and its size was consistent with the expected size of 477 bp deduced from the hypothalamic sequence (Adelman *et al.* 1986). To characterize these fragments further, we determined the nucleotide sequence of the

Figure 2 Agarose (1.2%) gel electrophoresis and Southern analysis of products obtained from RT-PCR of RNA extracted from testes (A), or ovaries (B), using GnRH primers. I, gel stained with ethidium bromide; II, Southern blot hybridization using ^{32}P -labeled oligonucleotide E; III, Southern blot hybridization using ^{32}P -labeled oligonucleotide i1. The expected sizes are indicated between A and B; M, DNA marker (HaeIII-digested PhiX-174); Ad, adult rat; P20, 20 day post-partum (dpp) rat; P4, 4 dpp rat; 14.5 to 21.5, stages of fetal development in days post-coitum (dpc); RT, control in which RNA was replaced by water during RT; PCR, control in which cDNA was replaced by water during PCR.

477 bp PCR product obtained from adult testis and investigated the presence of intronic sequences in the 1280 bp amplified fragments. We found a complete homology between the adult 477 bp GnRH cDNA sequence in the testis and that in the hypothalamus (Adelman *et al.* 1986). The hybridization of the 1280 bp fragment with [^{32}P]-labeled intronic probes revealed that i1 (panel A-III), but not i2 or i3 (data not shown), recognized this PCR product, the size of which was consistent with the conservation of the first intronic region (800 bp) in addition to the 477 bp amplified fragment (Bond *et al.* 1989).

In fetal testes (panel A-I, lanes 14.5 to 21.5) as well as in fetal ovaries (panel B-I, lanes 15.5 to 21.5), the gel analysis revealed a major fragment having the expected size of 477 bp. In addition, the 1280 bp fragment was visualized as a very weak signal in all the fetal testes (panel A-I, lanes 14.5 to 21.5) but not in the fetal ovaries (panel B-I, lanes 15.5 to 21.5). Its identity was confirmed after hybridization with E and i1 as described above (testes: panels A-II and A-III; ovaries: panels B-II and B-III). All signals were readily apparent after a longer time of exposure (data not shown).

Identification of GnRH-R cDNA in fetal gonads

Parallel amplifications of cDNA obtained from the same fetal gonads were performed using F2-R2. Gel electrophoresis of the products is represented in Fig. 3. A single fragment of about 1040 bp consistent with the expected

size deduced from the adult gonadal cDNA sequence (Moumni *et al.* 1994) was visualized by ethidium bromide, for all fetal testes (panel I-A, lanes 14.5 to 21.5) and ovaries (panel I-B, 15.5 to 21.5), but not for negative controls (lanes RT and PCR). The identity of this fragment was confirmed after hybridization to the internal [^{32}P]-labeled probe J (panel II-A: testes, panel II-B: ovaries).

Evolution of cyclophilin mRNA levels in fetal gonads

The amount of cyclophilin mRNA present in the total RNA extracted from fetal gonads is represented as a function of fetal stage in Fig. 4. Since all filters were processed together for hybridization with the cyclophilin probe and exposure to X-ray film, the results were expressed as arbitrary units. The cyclophilin mRNA levels are in the range of 0.04 arbitrary units for fetal testes, and 0.025 arbitrary units for fetal ovaries. In both cases, values fitted a linear curve of regression with a slope and a correlation coefficient close to zero ($P > 0.05$).

Evolution of GnRH and GnRH-R mRNA in gonads during fetal development

Densitometric data obtained for GnRH and GnRH-R mRNA were analyzed after standardization with cyclophilin, and expressed as arbitrary units. Since RNA extracted from fetal testes and ovaries were loaded onto the same filter and hybridized together with the same labeled probes, GnRH and GnRH-R gene expression were

Figure 3 Agarose (1.2%) gel electrophoresis and Southern analysis of products obtained from RT-PCR of RNA extracted from fetal rat testes (A) and ovaries (B), using GnRH-R primers. I, gel stained with ethidium bromide; II, Southern blot hybridization using ^{32}P -labeled oligonucleotide J. The expected size is indicated between A and B. M, DNA marker (HaeIII-digested PhiX-174); 14.5 to 21.5, fetal stages in days post-coitum; RT, control in which RNA was replaced by water during RT; PCR, control in which cDNA was replaced by water during PCR.

compared between the ovary and the testis, and during fetal development. No signal was found for the negative control RNA (stomach and limb buds), demonstrating the specificity of the hybridization with the cDNA probes. The variations in the GnRH (filter 1) and GnRH-R (filter 2) mRNA levels during fetal life are presented in Fig. 5. After stripping and rehybridization of filters (filter 1 with GnRH-R and filter 2 with GnRH cDNA probes), similar results were obtained (data not shown).

Some discrepancies appeared between the intensity of PCR signals and the dot blot results; however these do not reflect the true situation. Indeed, the RT-PCR protocol was not quantitative and the amplification may differ among the different samples, a difference that also appears on gel after amplification and on autoradiography after hybridization (Fig. 3). In contrast, the dot blot analysis is quantitative, and all values have been standardized to

cyclophilin. In fetal testes, both GnRH and GnRH-R mRNA were detected as early as 14.5 dpc, consistent with our RT-PCR data. During early development until 19.5 dpc, testicular GnRH mRNA levels fluctuated around 0.3 arbitrary units, then increased markedly. GnRH-R mRNA levels followed a similar pattern with slight fluctuations until 19.5 dpc. During the last two days of gestation, both mRNAs increased dramatically.

In fetal ovaries, in contrast to our results with RT-PCR, no GnRH mRNA was detected by dot blot hybridization until 18.5 dpc. GnRH mRNA levels increased markedly between 17.5 and 18.5 dpc, reaching a maximum of about 1.25 arbitrary units at 19.5 dpc then maintaining a plateau until term. As in the testes, low levels of GnRH-R mRNA were detectable at 15.5 dpc and then progressively increased until 19.5 dpc, after which they paralleled GnRH mRNA levels until birth.

Figure 4 Cyclophilin mRNA levels in rat fetal testes (A) and ovaries (B). Total RNA from fetal gonads was assessed for its cyclophilin mRNA content by dot blot hybridization using a ^{32}P -labeled cyclophilin cDNA probe. Autoradiograms were analyzed by densitometry. Data were standardized against the amount of total RNA loaded. Densitometric values expressed in arbitrary units (a.u.) are the results of two determinations of three diluted aliquots. Data were fitted to a linear regression with a slope and a correlation coefficient which were not statistically different from zero ($P > 0.05$).

Figure 5 Ontogeny of GnRH and GnRH-R mRNA in fetal rat gonads. Total RNA from fetal testes (◇) and fetal ovaries (■) was analyzed for its GnRH (A) and GnRH-R (B) mRNA content by dot blot hybridization to 32 P-labeled GnRH and GnRH-R cDNA probes followed by densitometry. Densitometric values obtained for three diluted aliquots were standardized with cyclophilin mRNA and expressed in arbitrary units (a.u.). Means (represented by short horizontal lines) were calculated and joined by connecting lines.

When gonadal GnRH and GnRH-R mRNA levels were compared between fetal testis and ovary, we noticed that GnRH and, to a lesser extent, GnRH-R mRNA levels appeared to increase earlier in the ovary than in the testis (18.5 vs 19.5 dpc). Furthermore, at 21.5 dpc the densitometric values for GnRH mRNA were equivalent in both sexes. By contrast, they were about 2.4 times higher in the testis than in the ovary after hybridization with the GnRH-R probe.

Discussion

The presence of GnRH receptors in Leydig cells (Clayton *et al.* 1980, Sharpe & Fraser 1980a) and in granulosa and luteal cells (Clayton *et al.* 1979, Harwood *et al.* 1980a,b, Reeves *et al.* 1980) has been supported by the identification of a mRNA with a sequence identical to that of the pituitary GnRH-R (Moumni *et al.* 1994). However, the exact nature of the endogenous GnRH-like material found in Sertoli cells (Sharpe & Fraser 1980b, Paull *et al.* 1981, Bhasin *et al.* 1983) and in granulosa cells (Aten *et al.* 1986) of adult rats remains uncertain.

In the present study using RT-PCR combined with Southern blotting, we have demonstrated the presence of a GnRH mRNA in the testis as well as in the ovary at all stages of development. After sequencing, we observed that adult rat testis transcript has a sequence identical to that of the hypothalamus (Adelman *et al.* 1986). These data suggest that one of the GnRH-like peptides previously described in the rat testis may correspond to authentic GnRH. Using the same protocol, Oikawa *et al.* (1990) discovered that authentic GnRH mRNA is present in rat ovary. The size of the PCR fragments obtained for fetal

and immature testes and ovaries, and their recognition by specific probes in Southern analysis, suggests that their sequence is also homologous to that of hypothalamic GnRH. In addition, we detected a larger sized PCR product in fetal, immature and adult testes which was absent in fetal ovaries. Southern blot hybridization with intronic probes demonstrated that this larger GnRH transcript retains the first intron. Such a transcript has been found in various human reproductive tissue and tumor cell lines: placenta (Seeburg & Adelman 1984, Dong *et al.* 1993, 1997), testis, ovary, prostate, mammary gland (Dong *et al.* 1993, 1997), and was found to be generated from a transcription start site located upstream from the usual site (Dong *et al.* 1993, 1997). As it was detected both in cytoplasmic and nuclear extracts of human placenta and mammary gland cell lines (Dong *et al.* 1993) it is more likely to be the result of a tissue-specific regulation of processing than a usual processing intermediate. Computer analysis (Marck 1988) of the rat gene sequence revealed that the presence of the first intron creates a translation site in phase, four codons upstream of the usual site. This suggests that this upstream site may initiate the synthesis of a proGnRH exhibiting a signal peptide elongated by four amino acids. The *in vitro* translation of the reproductive GnRH mRNA, described recently by Dong *et al.* (1997) in human, is compatible with the translational capacity of the transcript retaining intron 1 that we found in rat testis. Nevertheless, whether this transcript is present in the cytoplasm, whether it is translated from the additional start site, and whether the elongated prohormone generated is further processed as the hypothalamic proGnRH, are questions which remain to be answered. However, we could not detect transcripts lacking the first exon but retaining introns 2 and 3 as described in the adult ovary

(Goubau *et al.* 1992) because these transcripts could not be amplified using our set of primers. Taken together, these results indicate the presence of at least two populations of GnRH transcripts in the ovary.

In previous binding studies, the presence of GnRH-R was detected in the testis beginning one day after birth (Huhtaniemi *et al.* 1985). However, Habert *et al.* (1991) have shown that a GnRH agonist can exert a negative action on testicular steroidogenesis as early as 17.5–18.5 dpc. The present RT-PCR study detected a GnRH-R amplified fragment with a size identical to that of the adult form (Moumni *et al.* 1994) and recognized by a pituitary GnRH-R cDNA probe. As suggested for the adult rat, these data strengthen the idea that locally produced GnRH could operate as a paracrine or autocrine factor in the regulation of fetal gonadal functions through a specific receptor.

A prerequisite for evaluating a role for gonadal GnRH during fetal life is to study the ontogeny of expression of the GnRH and GnRH-R genes, with regard to age-dependent changes in cellular contents and in steroidogenesis. To this purpose, we investigated the presence of GnRH and GnRH-R mRNA in rat testis and ovary from 14.5 and 15.5 dpc respectively, when the morphological determination of the gonad is just completed, and in the testis, particularly when steroidogenic fetal Leydig cells would be differentiated (Bykov & Hoyer 1994, Ojeda & Urbanski 1994). We quantified GnRH and GnRH-R mRNA levels by dot blot hybridization of total RNA because this technique offers the advantages of comparing mRNA concentrations among different samples within one experiment, eliminating the variations in RNA loading by standardization to the same internal control. Densitometric values obtained with a labeled cyclophilin cDNA probe and standardized to total RNA, exhibited only slight fluctuations in the testis as well as in the ovary during fetal development, as attested by the regression analysis. This is consistent with the lack of regulation of cyclophilin expression in adult tissues (Danielson *et al.* 1988) and in the hypothalamus during postnatal development (Jakubowski *et al.* 1991). Thus, we decided that cyclophilin mRNA was an appropriate internal standard that could also be useful for future studies in fetal gonads.

Using RT-PCR and dot blot hybridization, we clearly demonstrated the presence of GnRH-R mRNA at 14.5 dpc in the testis and at 15.5 dpc in the ovary. By contrast, GnRH mRNA levels were quantified by dot blot from 18.5 dpc in the ovary, four days later than in the testis. However, using RT-PCR, GnRH mRNA was detected at 15.5 dpc. Such discrepancy between the dot blot and RT-PCR results may be explained by the high sensitivity of RT-PCR which allows the detection of mRNA in quantities under the limits of detection by dot blots, although the RT-PCR protocol we used in this study was not quantitative. Our present study revealed other differences between the ovary and the testis. In late

development, the GnRH-R mRNA concentration was about 2.4 times higher in the testis than in the ovary. In contrast, in the adult, the GnRH-R mRNA content was lower in the testis than in the ovary (respectively 5% and 40% compared with the pituitary) (Kakar *et al.* 1994). This suggests a postnatal switch in the level of expression of the GnRH-R gene between the sexes. In addition, we found that while GnRH and GnRH-R mRNA concentrations follow a concomitant increase in the gonads, this increase appears to occur earlier in the ovary than in the testis (18.5 dpc vs 19.5 dpc). Taken together, these results demonstrate that in rat gonads, the ontogeny of expression of the GnRH and GnRH-R genes is coordinately regulated but in a sex-dependent manner.

When compared with the developmental onset of gonadotropin receptor (LH-R and FSH-R) gene expression (Sokka *et al.* 1992, Zhang *et al.* 1994, Rannikki *et al.* 1995) LH-R transcripts appeared one day after GnRH-R mRNA in Leydig cells (15.5 and 14.5 dpc respectively), whereas FSH-R transcripts were detected in Sertoli cells two days later than GnRH mRNA (16.5 and 14.5 dpc respectively). In the ovary, gonadotropin receptor genes were expressed post-partum in granulosa cells. Thus, the onset of GnRH and GnRH-R gene expression in fetal gonads may be independent of LH-R and FSH-R gene expression in the corresponding cells, and in all cases, gonadal expression of the GnRH and GnRH-R genes appeared to precede, and thus also to be independent of, the initiation of LH and FSH secretion from the pituitary (Huhtaniemi 1995).

Treatment with GnRH agonists *in vivo* and *in vitro* have demonstrated an inhibitory effect of GnRH on Leydig cell steroidogenesis in immature and adult hypophysectomized rats (Bambino *et al.* 1980), and in decapitated rat fetuses (Habert 1992). In organotypic cultures of fetal testis, Habert *et al.* (1991) have also shown that a GnRH agonist inhibited basal testosterone production between 17.5–18.5 dpc and 20.5 dpc, and LH-stimulated testosterone production thereafter. These results suggest that GnRH-R mRNA is translated into a functional receptor protein very soon after its formation, but this cannot be verified by immunodetection, as antibodies against GnRH-R are not available at the present time. It is well documented that, *in vivo*, after reaching its maximum around 18.5 dpc, testosterone production by the testis remains unchanged (Warren *et al.* 1984), or decreases when it is expressed per cell (Picon 1976, Tapanainen *et al.* 1984, Saez 1994). During the same period, the density of cells producing testosterone per testis has been reported to be constant (Tapanainen *et al.* 1984, Kerr & Knell 1988, Saez 1994). Our finding of a marked increase in GnRH-R mRNA concentration suggests a stimulation of GnRH-R gene expression or an increase in the stability of GnRH-R mRNA. The decreasing steroidogenic activity of the Leydig cells is puzzling because of the concomitant rise in both the plasma concentration of LH (Huhtaniemi

1995) and the LH-R content (Warren *et al.* 1984). One possible explanation for this paradox is that some inhibitory paracrine/autocrine action may be established during late fetal life. The relationship between the marked increase in GnRH, as well as in GnRH-R, mRNA concentrations from 19.5 dpc to birth, and the decrease in Leydig cell activity, would be consistent with a potential implication of locally synthesized GnRH in such an inhibitory action. In addition, in cultured interstitial cells from the testes of hypophysectomized adult rats, GnRH agonists decrease testosterone production by an inhibitory effect on 17 α -hydroxylase and 17,20-desmolase activities (Hsueh *et al.* 1983). In the fetal testis, the onset of the main steroidogenic enzyme genes expression, and particularly of 17 α -hydroxylase, occurs around 15.5 dpc (Majdic *et al.* 1996), and thus early steroidogenesis, as GnRH and GnRH-R genes expression, is independent of gonadotropins (El-Gehani *et al.* 1998). We also point out that in the mouse, a species that lacks the gonadal GnRH-R (Wang *et al.* 1983), there is no decline in testosterone production in late fetal life (O'Shaughnessy *et al.* 1998).

In the adult rat ovary, *in vivo* and *in vitro* studies have demonstrated that, as in the testis, GnRH or GnRH agonists can affect gonadal functions. Indeed, GnRH has been shown to inhibit the expression of genes involved in steroidogenesis (Lynch *et al.* 1993, Richards 1994), and to modulate follicular response to gonadotropins, depending on the functional state of differentiation of follicular cells (Richards *et al.* 1987, Richards 1994, Srivastava *et al.* 1995). The autocrine actions of locally produced GnRH on steroidogenesis and/or folliculogenesis are difficult to assess in the fetal ovary, because these functions start a few days post-partum (Ojeda & Urbanski 1994, Huhtaniemi 1995). Moreover, there are no data reporting GnRH binding activity in the fetal ovary. In fact, granulosa cells are not yet organized in follicles (Byskov & Hoyer 1994, Ojeda & Urbanski 1994). However, as early as 13.5 dpc, the apparent precursors of theca and/or granulosa cells are able constitutively to express truncated LH-R mRNA (Sokka *et al.* 1996). Whether GnRH and GnRH-R mRNA are colocalized with these transcripts awaits further studies.

In conclusion, our study has demonstrated that the GnRH and GnRH-R genes are expressed in fetal gonads. The ontogeny of this expression underlined several similarities and differences between the sexes that encourage further investigation of the factors regulating GnRH and GnRH-R gene expression in gonadal tissues. Furthermore, our findings strengthen the previous hypothesis that gonadal GnRH could be involved in the age-related decrease of fetal Leydig cell activity and, post-partum, in the control of follicle differentiation in the ovary. Complementary studies, especially the ontogeny of expression of the GnRH and GnRH-R genes in postnatal gonads, will help us to evaluate further the role of GnRH as an intragonadal regulator.

Acknowledgements

We are indebted to Dr Lawrence Paul Aggerbeck (Centre de Génétique Moléculaire, CNRS UPR 2420, Gif sur Yvette, France) and to Dr Lisa Oliver (Institut de Biologie, INSERM U 419, Nantes, France) for correction of the english text. We wish to express our warmest thanks to Ms Anne-Marie Mathieu (Institut des Neurosciences, CNRS URA 1199, Paris, France) for her help in densitometry analysis of dots and for giving free access to the performant Autorad radioimaging device. We are grateful to Dr Bernard Vigier for kindly teaching us the dissection of fetuses and for helpful discussions. The expert assistance of Mrs Pierrette Thouvenot is also gratefully acknowledged.

References

- Adelman JP, Mason AJ, Hayflick JS & Seeburg PH 1986 Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. *Proceedings of the National Academy of Sciences of the USA* **83** 179–183.
- Arimura A, Serafini P, Talbo S & Schally AV 1979 Reduction of testicular luteinizing hormone/human gonadotropin receptors by (p-Trp⁶)-luteinizing hormone-releasing hormone in hypophysectomized rats. *Biochemical and Biophysical Research Communications* **90** 687–691.
- Aten RF, Williams AT, Behrman HR & Wolin DL 1986 Ovarian gonadotropin-releasing hormone-like protein(s): demonstration and characterization. *Endocrinology* **118** 961–967.
- Bambino TH, Schreiber JR & Hsueh AJW 1980 Gonadotropin-releasing hormone and its agonist inhibit testicular luteinizing hormone receptor and steroidogenesis in immature and adult hypophysectomized rats. *Endocrinology* **107** 908–917.
- Bhasin S, Heber D, Peterson M & Swerdloff R 1983 Partial isolation and characterization of testicular GnRH-like factors. *Endocrinology* **112** 1144–1146.
- Bond CT, Hayflick JS, Seeburg PH & Adelman JP 1989 The rat gonadotropin-releasing hormone SH locus: structure and hypothalamic expression. *Molecular Endocrinology* **3** 1257–1262.
- Byskov AG & Hoyer PE 1994 Embryology of mammalian gonads and ducts. In *The Physiology of Reproduction*, pp 487–540. Eds E Knobil & JD Neill. New York: Raven Press.
- Chomczynski P & Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162** 156–159.
- Clayton RN, Harwood JP & Catt KJ 1979 Gonadotropin-releasing hormone analogue binds to luteal cells and inhibits progesterone production. *Nature* **282** 90–92.
- Clayton RN, Katikineni M, Chan V, Dufau ML & Catt KJ 1980 Direct inhibition of testicular function by gonadotropin-releasing hormone: mediation by specific gonadotropin-releasing hormone receptors in interstitial cells. *Proceedings of the National Academy of Sciences of the USA* **77** 4459–4463.
- Danielson PE, Forss-Petter S, Brow MA, Calavetta L, Douglass J, Milner RJ & Sutcliffe JG 1988 p1B15: a cDNA clone of the rat mRNA encoding cyclophilin. *DNA* **7** 261–267.
- Dong KW, Yu KL & Roberts JL 1993 Identification of a major up-stream transcription start site for the human progonadotropin-releasing hormone gene used in reproductive tissues and cell lines. *Molecular Endocrinology* **7** 1654–1666.
- Dong KW, Yu KL, Chen ZG, Chen YD & Roberts JL 1997 Characterization of multiple promoters directing tissue-specific expression of the human gonadotropin-releasing hormone gene. *Endocrinology* **138** 2754–2762.

- El-Gehani F, Zhang F-P, Pakarinen P, Ranniko A & Huhtaniemi I 1998 Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biology of Reproduction* **58** 116–123.
- Eskey RL, Mical RS & Porter JC 1977 Relationship between luteinizing hormone-releasing hormone concentration in hypophyseal portal blood and luteinizing hormone release in intact, castrated, and electrochemically stimulated rats. *Endocrinology* **100** 263–270.
- Garrel G, Lerrant Y, Ribot G & Counis R 1993 Messenger ribonucleic acids for α - and β -isoforms of cyclic adenosine 3',5'-monophosphate-dependent protein kinase subunits present in the anterior pituitary. Regulation of RII β and Ca gene expression by the cyclic nucleotide and phorbol ester. *Endocrinology* **133** 1010–1019.
- Goubau S, Bond CT, Adelman JP, Misra V, Hynes MF, Schultz GA & Murphy BD 1992 Partial characterization of the gonadotropin-releasing hormone (GnRH) gene transcript in the rat ovary. *Endocrinology* **130** 3098–3100.
- Habert R 1992 Effect of decapitation and chronic *in vivo* treatment with a gonadotrophin-releasing hormone agonist on testicular steroidogenesis in the rat fetus. *Journal of Endocrinology* **133** 245–251.
- Habert R, Devif I, Gangnerau MN & Lecerf L 1991 Ontogenesis of the *in vitro* response of rat testis to gonadotropin-releasing hormone. *Molecular and Cellular Endocrinology* **82** 199–206.
- Harwood JP, Clayton RN & Catt KJ 1980a Ovarian gonadotropin-releasing hormone receptors. I. Properties and inhibition of luteal cell function. *Endocrinology* **107** 407–413.
- Harwood JP, Clayton RL, Chen TT, Knox G & Catt KJ 1980b Ovarian gonadotropin-releasing hormone receptors. II. Regulation and effects on ovarian development. *Endocrinology* **107** 414–421.
- Hsueh AJW & Erickson GF 1979 Extrahypothalamic inhibition of testicular function by luteinizing hormone-releasing hormone. *Nature* **281** 66–67.
- Hsueh AJW & Jones PBC 1981 Extrahypothalamic actions of gonadotropin-releasing hormone. *Endocrine Reviews* **2** 437–461.
- Hsueh AJW, Bambino TH, Zhuang L-Z, Welsh TH & Ling NC 1983 Mechanism of the direct action of gonadotropin-releasing hormone and its agonist on androgen biosynthesis by cultured rat testicular cells. *Endocrinology* **112** 1653–1661.
- Huhtaniemi I 1995 Molecular aspects of the ontogeny of the pituitary-gonadal axis. *Reproduction, Fertility and Development* **7** 1025–1035.
- Huhtaniemi IT, Catt KJ & Clayton RN 1985 Newborn and immature rat testes contain gonadotropin-releasing hormone (GnRH) receptors, and their testosterone production is stimulated by a GnRH agonist *in vitro*. *Molecular and Cellular Endocrinology* **40** 41–44.
- Jakubowski M, Blum M & Roberts JL 1991 Postnatal development of gonadotropin-releasing hormone and cyclophilin gene expression in the female and male rat brain. *Endocrinology* **128** 2702–2708.
- Kakar SS, Grantham K, Musgrove LC, Devor D, Sellers JC & Neill JD 1994 Rat gonadotropin-releasing hormone (GnRH) receptor: tissue expression and hormonal regulation of its mRNA. *Molecular and Cellular Endocrinology* **101** 151–157.
- Kerr JB & Knell CM 1988 The fate of fetal Leydig cells during the development of the fetal and postnatal rat testis. *Development* **103** 535–544.
- Lefebvre FA, Reeves JJ, Seguin C, Massicotte J & Labrie F 1980 Specific binding of a potent LHRH agonist in rat testis. *Molecular and Cellular Endocrinology* **20** 127–134.
- Lynch JP, Lala DS, Peluso J, Luo W, Parker KL & White BA 1993 Steroidogenic factor 1, an orphan receptor, regulates the expression of the rat aromatase gene in gonadal cells. *Molecular Endocrinology* **7** 776–786.
- Majdic G, Sharpe RM, O'Shaughnessy PJ & Saunders PTK 1996 Expression of cytochrome P450 17 α -hydroxylase/C17–20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* **137** 1063–1070.
- Marck C 1988 DNA Strider: a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. *Nucleic Acids Research* **16** 1829–1836.
- Moumni M, Kottler ML & Counis R 1994 Nucleotide sequence analysis of mRNA predicts that rat pituitary and gonadal gonadotropin-releasing hormone receptor proteins have identical primary structure. *Biochemical and Biophysical Research Communications* **200** 1359–1366.
- Nett TM, Akbar AM & Niswender GD 1974 Serum levels of luteinizing hormone and gonadotropin-releasing hormone in cycling, castrated and anoestrous ewes. *Endocrinology* **94** 713–718.
- Oikawa M, Dargatzis C, Ny T & Hsueh AJW 1990 Expression of gonadotropin-releasing hormone and prothymosin- α messenger ribonucleic acid in the ovary. *Endocrinology* **127** 2350–2356.
- Ojeda SR & Urbanski HF 1994 Puberty in the rat. In *The Physiology of Reproduction*, pp 363–409. Eds E Knobil & JD Neill. New York: Raven Press.
- O'Shaughnessy PJ, Baker P, Sohnius U, Haavisto A-M, Charlton HM & Huhtaniemi I 1998 Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology* **139** 1141–1146.
- Paull WK, Turkelson CM, Thomas CR & Arimura A 1981 Immunohistochemical demonstration of a testicular substance related to luteinizing hormone-releasing hormone. *Science* **213** 1263–1264.
- Picon R 1976 Testosterone secretion by foetal rat testes *in vitro*. *Journal of Endocrinology* **71** 231–238.
- Rannikko AS, Zhang FP & Huhtaniemi IT 1995 Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. *Molecular and Cellular Endocrinology* **107** 199–208.
- Reeves JJ, Seguin C, Lefebvre FA, Kelly PA & Labrie F 1980 Similar luteinizing hormone-releasing hormone binding sites in rat anterior pituitary and ovary. *Proceedings of the National Academy of Sciences of the USA* **77** 5567–5571.
- Reinhart J, Xiao S, Arora KK & Catt KJ 1997 Structural organisation and characterization of the promoter region of the rat gonadotropin-releasing hormone receptor gene. *Molecular and Cellular Endocrinology* **130** 1–12.
- Richards JS 1994 Hormonal control of gene expression in the ovary. *Endocrine Reviews* **15** 725–751.
- Richards JS, Jahnsen T, Hedin L, Lifka J, Ratoosh S, Durica JM & Goldring NB 1987 Ovarian follicular development: from physiology to molecular biology. *Recent Progress in Hormone Research* **43** 231–276.
- Saez JM 1994 Leydig cells: endocrine, paracrine, and autocrine regulation. *Endocrine Reviews* **15** 574–626.
- Sambrook D, Fritsch EF & Maniatis T 1989 *Molecular Cloning, a Laboratory Manual*. New-York: Cold Spring Harbor Laboratory.
- Seeburg PH & Adelman JP 1984 Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone. *Nature* **311** 666–668.
- Sharpe RM & Fraser HM 1980a Leydig cells receptors for luteinizing hormone-releasing hormone and its agonists and their modulation by administration or deprivation of the releasing hormone. *Biochemical and Biophysical Research Communications* **95** 256–262.
- Sharpe RM & Fraser HM 1980b hCG stimulation of testicular LHRH-like activity. *Nature* **287** 642–643.
- Sharpe RM, Doogan DG & Cooper I 1982 Stimulation of Leydig cell testosterone secretion *in vitro* and *in vivo* in hypophysectomized rats by an agonist of luteinizing hormone releasing hormone. *Biochemical and Biophysical Research Communications* **106** 1210–1217.
- Sokka TA, Hämäläinen TM & Huhtaniemi IT 1992 Functional LH receptor appears in the neonatal rat ovary after changes in the alternative splicing pattern of the LH receptor mRNA. *Endocrinology* **130** 1738–1740.
- Sokka TA, Hämäläinen TM, Kaipia A, Warren DW & Huhtaniemi IT 1996 Development of luteinizing hormone action in the perinatal ovary. *Biology of Reproduction* **55** 663–670.

- Srivastava RK, Krishna A & Sridaran R 1995 Follicular development, steroidogenesis and gonadotrophin secretion in response to long-term treatment with a gonadotrophin-releasing hormone agonist in the rat. *Journal of Endocrinology* **146** 349–357.
- Tapanainen J, Kuopio T, Pelliniemi IJ & Huhtaniemi IT 1984 Rat testicular endogenous steroids and number of Leydig cells between the fetal period and sexual maturity. *Biology of Reproduction* **31** 1027–1035.
- Wang NG, Sundaram K, Pavlou S, Rivier J, Vale W & Bardin CW 1983 Mice are insensitive to the antitesticular effects of luteinizing hormone-releasing hormone agonists. *Endocrinology* **112** 331–335.
- Warren DW, Huhtaniemi IT, Tapanainen J, Dufau ML & Catt KJ 1984 Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology* **114** 470–476.
- Zhang FP, Hämäläinen TM, Kaipia A, Pakarinen P & Huhtaniemi IT 1994 Ontogeny of luteinizing hormone receptor gene expression in the rat testis. *Endocrinology* **134** 2206–2213.

Received 23 February 1998
Accepted 9 June 1998