Delayed expression of the insulin-like growth factor I (IGF-I) gene in the XY sex-reversed female mouse ovary

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ABSTRACT   When the Y chromosome of *Mus musculus domesticus* (Y^{DOM}) mouse strain from Tirano (Italy) or *Mus musculus poschiavinus* (Y^{POS}) from Poschiavo (Switzerland), is placed onto the C57BL/6J (B6) inbred background, the Y^{DOM} chromosome fails to induce normal testicular differentiation and instead allows development of ovaries and ovotestes in embryonic life. During postnatal development some hermaphroditic males become fertile whereas the XY females lack normal estrus cyclicity, produce low levels of gonadal steroids and cannot carry pregnancy to term. Here we studied the transcription of the IGF-I gene known to be involved in steroidogenesis. RNA was isolated from the XX and the XY ovaries at 1 to 40 d.p.p. and subjected to RT-PCR analysis. Immunocytochemical staining for IGF-I was performed to identify the cell type of IGF-I peptide localization, and protein expression was examined by Western blot analysis. The present results indicate that the IGF-I transcript was expressed at 1 d.p.p. in the XX ovary throughout the studied stages whereas in the XY ovary mRNA IGF-I was not detected until 15 d.p.p. IGF-I protein was identified in theca cells in the whole XX control ovary, while in the XY ovary, strong staining for IGF-I was found in the theca cells of the cortex. Faint staining was also seen around the medullary sterile cords. Western blot analysis showed normal onset in the XX and the XY ovary, but a different staining pattern for IGF-I in the XY ovary at 11 and 26 d.p.p. compared to the XX control ovary. We propose that delayed expression of IGF-I in the XY mouse ovary may be responsible for low steroid production and fertility problems in the XY sex-reversed adult female mouse.

KEY WORDS: *insulin-like growth factor I, fertility, ovary, sex-reversal, mouse*

Introduction

Gonadal differentiation is a cascade of molecular, morphological and biochemical events that must occur at the appropriate time and sequence during embryonic development to induce male or female gonadal sex differentiation and the associated phenotype. Male differentiation is determined by the testis determining factor named SRY(Sry) gene on the Y chromosome (Berta *et al.*, 1990; Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Koopman *et al.*, 1991). Nucleotide and aminoacid sequence homology to the high mobility proteins group suggested that the Sry gene product is a transcription factor that controls a regulatory cascade of genetic events leading to testicular differentiation (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990). However, the mechanism(s) still remain(s) to be discovered.

On the other hand, when the Y chromosome of *Mus musculus domesticus* mouse strain from Tirano (Italy) or *Mus musculus poschiavinus* from Poschiavo (Switzerland) is placed onto the C57BL/6J (B6) background, the XY progeny (B6.Y^{DOM}) develop ovaries and ovotestes, but never normal testes in embryonic life (Eicher *et al.*, 1982; Eicher and

Washburn, 1983, 1986). Eicher and Washburn (1986) proposed that the *Tdy* allele of Y^{DOM} initiates testis differentiation later than the *Tdy* of B6. This hypothesis was further supported by Taketo *et al.* (1991) and Palmer and Burgoyne (1991) who studied the onset of testicular differentiation in different mouse genetic backgrounds. We previously demonstrated that during postnatal development some hermaphroditic males become fertile while the XY females lack normal estrus cyclicity (Taketo-Hosotani *et al.*, 1989), produce low levels of gonadal steroids (Villalpando *et al.*, 1993) and fail to produce litters, due to the death of most embryos at very early stages (Merchant-Larios *et al.*, 1994).

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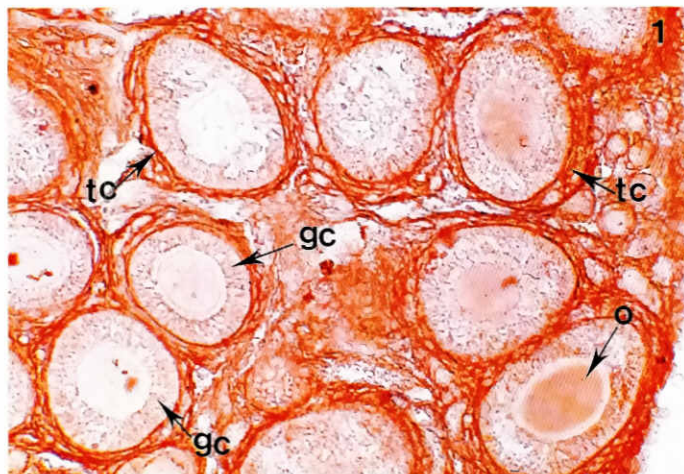
Fig. 1. Immunocytochemical staining for IGF-I in the XX B6.Y^{DOM} ovary. *Intense staining of IGF-I was observed in whole XX ovary mainly in the theca cells (tc) around the follicles at 14 d.p.p. Some oocytes (o) and some granulosa cells (gc) of developing follicles were only faintly stained. x400.*

Recently, it has been shown that the Sry gene expression was prolonged one more day in the B6.Y^{DOM} gonads than in the normal testis and that biochemical products of embryonic testes, such as MIS, 3ß-HSD and 17 α-HSD were absent or transiently detected in the B6.Y^{DOM} ovary. The P450arom gene that converts androgen to estrogen was transcribed in both XX and XY gonads (Lee and Taketo, 1994) at the onset of gonadal differentiation. These results suggest that the testis determining pathway in the XY ovary appears to be impaired downstream of Sry gene expression.

Ovarian growth and development is characterized by a dramatic increase and differentiation of follicles destined to ovulate. Gonadotropins play an important role in the regulative fashion of these events (Richards *et al.*, 1987). Granulosa cell proliferation and differentiation seem to be regulated *in vitro* by intraovarian factors, among them insulin-like growth factor-I. It has been proposed that IGF-I is likely to act as a paracrine or/and autocrine factor in the ovary (Adashi *et al.*, 1985, 1990; Magoffin *et al.*, 1990), but the mechanism remains poorly understood.

Granulosa cells are the site of production and/or reception of IGF-I (Oliver *et al.*, 1989). This peptide apparently synergizes with gonadotropins to increase gonadal steroids production *in vitro* in several species of mammals, such as humans (Bergh *et al.*, 1991; Christman *et al.*, 1991), swine (Baranao and Hammond, 1984; Hsu and Hammond, 1987; Morley *et al.*, 1989; Gooneratne *et al.*, 1990; Randall *et al.*, 1990), rabbits (Cassandra *et al.*, 1991), marmosets (Shaw *et al.*, 1993) and rats (Hammond *et al.*, 1985; Hernandez *et al.*, 1988; Carlsson *et al.*, 1989; Oliver *et al.*, 1989; Adashi *et al.*, 1990). Most research on IGF-I has been performed using the rat as a biological model and little is known about its role in the physiology of mouse ovary. We examined here the ontogeny of IGF-I gene expression and its immunoreactivity in the XX and the XY sex-reversed mouse ovary during postnatal development. Results show that the molecular size of IGF-I positive protein bands are different between the XX and the XY ovary and that mRNA

IGF-I gene expression is delayed in the XY ovary compared to the XX normal litter mate ovary. We propose that delayed expression of the IGF-I gene can partially explain low levels of gonadal steroids and the fertility problems in the XY adult female mouse.

Results

Ontogeny of IGF-I staining in the B6.Y^{DOM}ovary

In the XX females at 14 d.p.p. we observed follicles at different developmental stages in whole ovary. At the same age in the XY sex-reversed mouse, ovary follicles were located in the cortex while in the medullary area the sterile cords were seen. These are formed by granulosa-like cells and theca-like cells (data not shown). We identified these cells with a battery of antibodies against intermediate filaments of epithelial and mesenchymal cells (unpublished data).

Faint staining for IGF-I was observed in the XX and the XY ovary at 1 to 12 d.p.p. which remained almost at background level (data not shown). Immunocytochemical staining for IGF-I in the XX ovary showed morphological characteristics of well organized follicles. Intense staining was seen in theca interstitial cells in the interstitium and in the theca cells around the follicles at different developmental stages in whole XX ovary at 14 d.p.p. Some oocytes of developing follicles in the XX ovary were moderately stained. Most granulosa cells of total follicle population remained unstained (Fig. 1). In the control sections where the anti-IGF-I was omitted and with preabsorbed antibody, no staining was seen neither in theca interstitial cells nor in the theca cell in the XX ovary at 14 d.p.p. (Fig. 2). In the XY ovary at 14 d.p.p. strong staining for IGF-I was visualized in theca interstitial cells and in the theca cells around the follicles located in the cortex region. Faint staining for IGF-I that remained at background level was seen in granulosa cells in this area. Moderate staining for
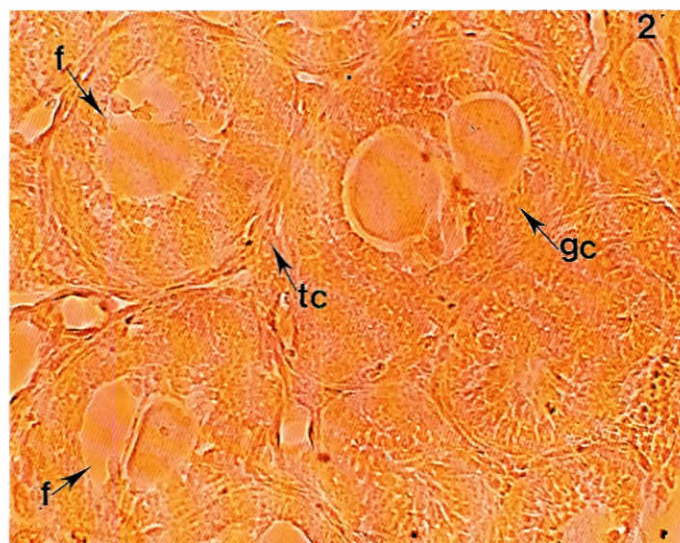
Fig. 2. Immunocytochemical staining for IGF-I in the XX B6.Y^{DOM} ovary. *In the XX B6.Y^{DOM} normal ovary at 14 d.p.p. where the first antibody was omitted and no immunoreactivity was observed in the theca (tc) and granulosa cells (gc) of follicles (f) at different developmental stages in whole ovary. x400.*
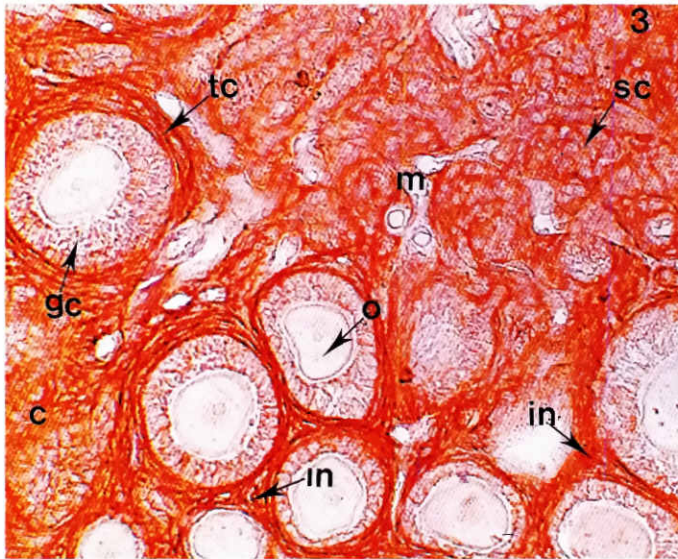
Fig. 3. Immunocytochemical staining for IGF-I in the XY B6.Y^DOM ovary. *Intense staining of IGF-I was observed in theca interstitial cells (in) and in the theca cells (tc) around the follicles located in the cortex (c) region of the XY ovary at 14 d.p.p. Faint staining was seen in some granulosa cells (gc) of follicles located in this area. Intense staining of IGF-I was also observed around the sterile cords of different sizes (sc) in the theca-like cells of the medulla (m). Most oocytes (o) remained unstained. x400.*

IGF-I was located around the sterile cords in theca-like cells and the interstitial cells in the medullary area (Fig. 3).

Immunoreactivity of IGF-I protein in the B6.Y^DOM gonad

Immunoreactivity of IGF-I protein examined by Western blot analysis was detected since 1 d.p.p. and its expression was similar in both the XX and the XY ovary. At this early developmental stage, two bands for IGF-I protein with apparent molecular weights of 45 and 38 kD respectively, were identified. The same staining pattern for this peptide was seen at 6 d.p.p. (Fig. 4). However, at 11 d.p.p. the XY ovary showed staining bands for IGF-I protein additional to those detected in the control XX ovary. Later during development at 26 d.p.p. IGF-I protein expression gave a different staining pattern in the XY ovary.

Ontogeny of IGF-I gene expression

The IGF-I messenger was detected since 1 d.p.p. in the control XX ovary and the same levels of mRNA were found throughout all stages studied (Fig. 5). No bands or faint bands were seen in the control which was analyzed by omitting the reverse transcriptase enzyme during cDNA synthesis of IGF-I. In the XY ovary, IGF-I gene expression was absent at 1, 5 and 10 d.p.p. IGF-I transcripts were not observed until 15 d.p.p. and their expression was maintained in all subsequently studied stages (Fig. 5).

Discussion

In the present study we found that the IGF-I mRNA was expressed in the XX normal ovary at 1 d.p.p. and its expression was maintained throughout all developmental stages studied,

whereas it was not detected until 15 d.p.p. in the XY ovary. Since IGF-I gene transcripts were seen at 1 d.p.p. at the onset of postnatal development, it is possible that the early presence of IGF-I is required as a signal during normal ovarian development to induce growth and expression of enzymes involved in steroid production. It has been shown that the mouse ovary at 1-5 d.p.p. is characterized by stage II of folliculogenesis in which oocytes are separated by epithelial cells inside the sex cords and, at 10-15 d.p.p., oocytes surrounded by epithelial cells and enveloping lamina basal are individualized by stromal tissue (Merchant-Larios and Chimal-Monroy, 1989). These follicles initiate growth by a signal not yet known. It is conceivable to postulate that delayed expression of IGF-I could be due to the impairment of early folliculogenesis in the XY ovary. We have previously demonstrated that the XY ovary starts to lose germ cells in the medullary area at 17 d.g. (Taketo-Hosotani et al., 1989). Atresia of follicles continues in such a way that only two or three follicles survive until 150 d.p.p.

Delay of IGF-I gene expression could also be attributed to a smaller amount of IGF-I messenger present at this particular age. Contrary to general thoughts, in this study RT-PCR was not sensitive enough to detect low levels of mRNA which alternatively could have been translated into protein as efficiently as in the XX ovary.

At present, we do not know if different transcript sizes of IGF-I are produced during ovarian development in the XX and XY female, but Northern blot analysis and IGF-I protein content of follicular complexes at different stages will be further investigated.

It is interesting that the mRNA and its protein were detected at 1 to 26 d.p.p. in the XX normal ovary when the hypothalamus-hypophysis-gonad axis is not yet established in rodents (Frawley
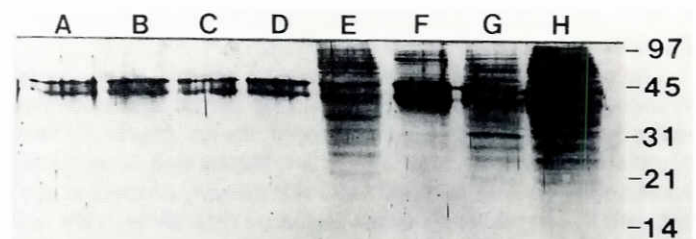
Fig. 4. Immunoreactivity for IGF-I protein analysis in the B6Y^DOM ovaries. *Two bands of IGF-I peptide were stained at 1 to 6 d.p.p. in both the XX (lanes B and D) and the XY ovary (lanes A and C). However, a different staining pattern and additional IGF-I bands were identified in the XY ovary at 11 d.p.p. (lane E) compared to the XX normal ovary (lane F). In the XY ovary (lane G) at 26 d.p.p. different bands for IGF-I were also expressed whereas stronger staining for this peptide and a different staining pattern was also visualized in the XX ovary at the same age (lane H). The molecular weights of standard proteins are indicated.*
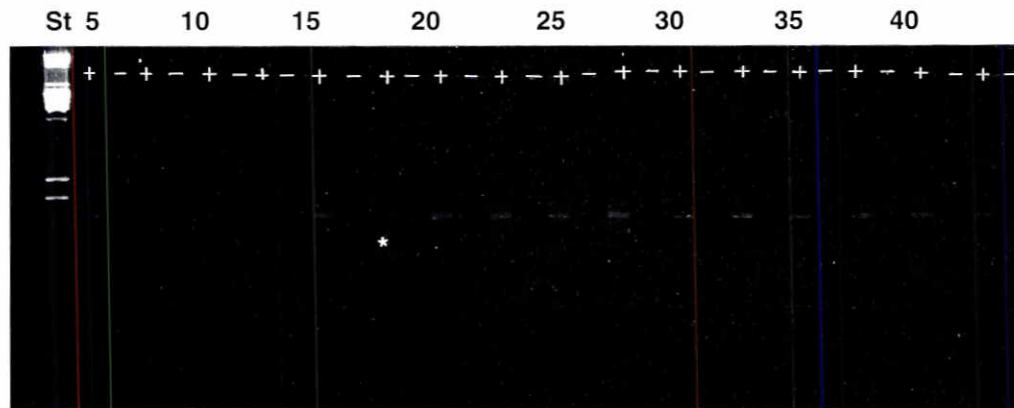
Fig. 5. Ontogeny of IGF-I expression in the B6Y^DOM gonads. *RT-PCR analysis was done in the presence (+) or absence (-) of reverse transcriptase. St indicates molecular weight standard and the expected fragment size was 212 bp. mRNA gene expression is always shown first in the XX ovary than in the XY ovary. IGF-I transcripts were seen in the XX ovary from 5 to 40 d.p.p., whereas in the XY ovary IGF-I expression was detected later, at 15 d.p.p. (asterisk), and its expression was maintained until 40 d.p.p.*

and Henricks, 1979; Richards *et al.*, 1987). These findings suggest that the early growth of follicles could be regulated by IGF-I independently of gonadotropins. The fact that the IGF-I protein was detected as early as 1 d.p.p. suggests that the proteins could have been transported from a non-ovarian source.

The IGF-I gene has distinct transcription start sites, alternative splicing sites to produce multiple E peptides, and multiple polyadenylation sites which lead to different IGF-I mRNA molecular weight species (Adamo *et al.*, 1994). It has been proposed that despite the presence of multiple mRNA IGF-I, only one mature peptide is synthesized (Rotwein *et al.*, 1993). In the present study, however Western blot analysis showed that the IGF-I protein is expressed since 1 d.p.p. in both the XX and the XY ovary and different IGF-I molecular weight bands were detected both before folliculogenesis (11 d.p.p.) and after initiation of this process (26 d.p.p.). These data suggest that various IGF-I peptides could be synthesized at these stages under stimuli different to hormonal control. It is also possible that one peptide is synthesized and modified posttranslationally.

The presence of high molecular weight bands cannot be due to unspecificity of the antibody used in the immunoblot since this antibody has been previously tested in rat neural system (García-Segura *et al.*, 1991). Protein degradation is excluded because the tissues obtained were immediately processed and because the same band pattern appeared repeatedly in the five trials of these experiments. Furthermore, the presence of these different bands of IGF-I in the XX and the XY ovary along folliculogenesis may reflect the different stages of follicular development.

IGF-I immunoreactivity was mainly identified in interstitial theca cells and theca cells of developing follicles in whole XX ovary at 14 d.p.p. while faint staining was seen in some granulosa cells. These findings demonstrate for the first time that the site of IGF-I localization in the mouse ovary are theca cells and not granulosa cells. Oliver *et al.* (1989) demonstrated that the granulosa cells are the only type which express the IGF-I gene in the rat ovary. Whether this conclusion applies to most species of vertebrates is uncertain. There are probably species-specific differences.

Faint staining for IGF-I observed in the medullary sterile cords at 14-25 d.p.p. may suggest that the levels of this peptide in the XY ovary could be lower than in the XX ovary. It is likely that a certain amount of IGF-I is necessary to induce steroid production *in vivo*.

We have previously demonstrated that the XY sex-reversed female mouse produces low levels of steroids (Villalpando *et al.*, 1993). This abnormality is not related to the malfunction of the hypophysis since ovariectomized XY female grafted with ovaries from the XX female litter mate can initiate estrus cyclicity. Reciprocally, the XX female that received XY ovarian grafts did not resume estrus cyclicity, suggesting that the problem of the XY female resides within the ovary. Our studies suggest that one of the impaired functions in the XY ovary is the late expression of IGF-I gene expression. Carlsson *et al.* (1989) examined ovarian levels of IGF-I mRNA and protein throughout the estrus cycle in the rat ovary. These authors found that the mRNA levels of IGF-I during estrus were higher than in proestrus. If IGF-I protein is of importance as a physiological modulator or mediator for the cyclic developmental changes in the ovary, it is possible that IGF-I should be cycle-dependent.

We propose that impaired early folliculogenesis could lead to delayed expression and/or lower production of IGF-I in the XY ovary, which in turn, could impair expression of gonadal steroid enzymes and, consequently, induce low levels of gonadal steroid production in the infantile ovary, leading to lack of normal estrus cyclicity and fertility problems in the XY adult female mouse.

Materials and Methods

Animal strain

Chromosomal sex

Chromosomal sex of individual XY females was determined by dot hybridization of peripheral blood from the tail with a mouse Y chromosome-specific DNA probe 145SC5 as described before (Nishioka, 1988).

Determination of protein content

Protein content of the ovaries was determined by the method described by Bradford (1976) using BSA as a standard and a Bio-Rad Protein Assay Kit.

Immunocytochemistry

B6.Y^DOM mouse ovaries were dissected and immediately fixed in 4% paraformaldehyde in 0.1 M PBS pH 7.4 at 4° C for 2 h. Ovaries were

washed twice with the same buffer and left in 7% sucrose in PBS overnight at 4°C. Tissues were dehydrated in 25-95% polyethylene glycol (PEG mol. wt 400) solutions at room temperature. After four changes of PEG (mol. wt 1000), tissues were embedded in PEG (mol. wt 1450). Serial sections of 10 μm thickness were obtained and 5-7 of them were floated in distilled water. Subsequently, sections were placed in small plastic chambers sealed with Nitex mesh. For the immunocytochemical stain we used an anti-IGF-I polyclonal antibody (anti-somatomedin-C/IGF-I rabbit antiserum NIH-UB-4, a kind gift of Drs. L. Underwood and J.J. Van Wyck, and obtained through the National Hormone and Pituitary Program of NIH, Bethesda). This antiserum has 0.5% cross reactivity with IGF-II and crossreacts minimally with insulin at 10^{-6} M (information provided by NIH). Sections were incubated with 0.1% Triton X-100 for 5 min and then placed in 2% bovine serum albumin for 20 min. Immunological detection was performed following the manufacturer's instructions of Vectastain ABC Kit (Vector Laboratories) with slight modifications. Peroxidase activity was revealed with 0.3% H_2O_2 using 3´3 diaminobenzidine tetrahydrochloride as a chromogen and washed in distilled water. After washing, 3-5 sections were placed on microscope slides and studied under a Nikon HFX.II microscope. Preabsorption of the NIH-UB-4 antibody for 2 h at 22°C with 10^{-7} M synthetic human IGF-I results in complete abolishment of immunostaining. Also primary antibody was omitted as negative control.

Western blot analysis

Both XX and XY B6.Y^{DOM} ovaries from 1-26 d.p.p. were homogenized in 0.2 M Tris-HCl, pH 6.8 with a Tri-R Stir-R homogenizer for 45 sec at 4°C and centrifuged at 1500 rpm for 5 min. Hereafter, the complete process was carried out at 4°C. After centrifugation, samples from the supernatant containing 10-20 μg of total proteins were loaded and analyzed by electrophoresis in a 15% polyacrylamide gel (Laemmli, 1970). Subsequently, proteins were transferred to PVDF membrane in 0.25 M Tris-HCl, 0.192 M glycine and 20% methanol at pH 8.3 (Towbin et al., 1979). The membrane was blocked with 3% serum bovine albumin overnight and incubated with anti IGF-I at a dilution 1:1000 for 24 h. After washing four times with 0.01 M Tris-HCl pH 7.4, 0.9% NaCl and 0.1% Tween 20 the membrane was incubated with peroxidase-horseradish goat anti-rabbit IgG at a 1:1000 dilution for 24 h. The reaction was revealed with 4 chloro-1-naphtol with 0.08% hydrogen peroxide according to the method described by Towbin et al. (1979).

PCR amplification

Total RNA was isolated from ovaries according to the method described by Chomczynski and Sachi (1987) and resuspended in 20 μl DEPC-treated water. Then 2 μl RNA samples containing 1 μg of RNA were denatured at 90°C for 5 min and subjected to cDNA synthesis in a total volume of 20 μl containing 200 units of M-MLV reverse transcriptase (Perkin Elmer Cetus, Roche Molecular Systems), 1 mM of each dNTPs (Perkin Elmer Cetus), 5 pmol of random hexamers (Perkin Elmer Cetus), 10 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$ and 50 mM KCl. Negative controls were performed without reverse transcriptase. The reaction mixture was incubated at 25°C for 10 min and subsequently at 42°C for 60 min. The enzyme was then inactivated at 99°C for 5 min. PCR amplification was performed with a Perkin Elmer Cetus Thermocycler (model 9600) with a set of sense and antisense IGF-I primers, 30 pmol of each, 2.5 units Taq DNA polymerase (Perkin Elmer Cetus), 10 mM Tris-HCl pH 8.3, 50 mM KCl and 3.5 mM $MgCl_2$ in a total volume of 100 μl. Total cycle numbers were 30; 28 cycles included denaturation of each at 94°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C. In the first cycle denaturation was performed for 2 min and in the last cycle extension was 10 min. The oligonucleotide sequence of IGF-I sense and antisense primers for PCR were designed according to Bell et al. (1986) and they were synthesized by the Unidad de Análisis y Aminoácidos, Instituto de Investigaciones Biomédicas, Universidad Nacional Autonóma de México. The expected fragment size for IGF-I produced by RT-PCR was 212 bp.

Then 10 μl of PCR amplification products of each reaction were separated in 1.5% agarose gel electrophoresis in Tris-acetate/EDTA buffer and visualized with ethidium bromide staining (Sambrook et al., 1989).

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