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FGF-8 Is Expressed during Specific Phases of Rodent Oocyte and Spermatogonium Development

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We have studied the localization of the expression of FGF-8 mRNA in adult and developing rat and mouse gonads by *in situ* hybridization. The expression of FGF-8 mRNA was high in oocytes of small and large antral follicles of adult mouse ovaries. No signal was observed in fetal ovaries, or in primordial and atretic follicles of adult ovary. In mouse testis, the FGF-8 mRNA signal could be demonstrated in prespermatogonia during a short period covering the fetal days 15 to 17, but not any more on day 19 of fetal life, or in adult testis. The time course of the expression of FGF-8 mRNA in mouse testis was confirmed by RT-PCR reaction. Corresponding *in situ* results were obtained by studying rat tissues. The observed germ cell-specific expression of FGF-8 mRNA in maturing oocytes and fetal prespermatogonia suggests that FGF-8, which is a secretory protein, has a paracrine function during the specific phases of the maturation of the follicle and fetal seminiferous epithelium. © 1997 Academic Press

INTRODUCTION

FGF-8 is a member of the mammalian fibroblast growth factor (FGF) family (1), which consists of ten members: acidic FGF/FGF-1, basic FGF/FGF-2, int-2/FGF-3, HST (k-FGF)/FGF-4, FGF-5, HST-2/FGF-6, KGF/FGF-7 (1), AIGF/FGF-8 (2), GAF/FGF-9 (3) and FGF-10 (4). They transduce signals through transmembrane receptor tyrosine kinases (5), and also show marked affinity to glycosaminoglycans and proteoglycans of cell surface and extracellular matrix (6).

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FGF-8 was initially identified as an androgen inducible growth factor (AIGF) in the SC-3 mammary carcinoma cell line from which it was first cloned (2). In the mouse genome, the FGF-8 locus has been mapped to the distal region of chromosome 19 (7). The human FGF-8 gene has been cloned from placental genomic library (8) and localized to chromosome 10 (9). The structure of FGF-8 gene is more complicated than that of the other members of the FGF family. Alternative splicing of the FGF-8 gene potentially allows eight protein isoforms, which differ in the amino terminus of the mature polypeptides (7,10). FGF-8 is a secreted protein which has been shown to have transforming activity *in vitro* (11) and, according to MacArthur *et al.* (1995) (12), the isoform FGF-8b has the highest potential to transform NIH3T3 cells.

FGF-8 is widely expressed in embryonal tissues. The expression shows a temporally and spatially restricted pattern at the sites that direct the outgrowth and patterning of the embryo (7,13,14). FGF-8 has apparently a specific function in the elongation of the body axis, morphogenesis of the central nervous system, limb, and face. Interestingly, however, in adult mouse FGF-8 mRNA expression has been detected by Northern blotting only in ovary and testis, but not in any other of the normal nonmalignant tissues studied (15).

This work was undertaken to study the *in vivo* expression of the FGF-8 mRNA in fetal and adult rodent ovary and testis by *in situ* hybridization. The results demonstrate a developmentally regulated expression of FGF-8 in rodent gonads.

MATERIALS AND METHODS

Experimental animals. Mouse (BalbC) fetuses at the age of 11-19 days and rat (Sprague-Dawley) fetuses at the age of 12-19 days, and 90-day-old adults were used in the *in situ* hybridization study. For the fetal age, the day after copulation was designated as the Day 0. Sex of 11-13 day mouse and 12-14 day rat fetuses was determined

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by X-chromatin staining of the cells from the amnion membrane. Sex of older fetuses was determined on the basis of the gonadal morphology. Adult mice and rats were housed at constant temperature (20°C) and light-dark cycle (lights 06.00-20.00). The animals had free access to food and water.

RNA extraction and Northern blot hybridization. Total RNA from adult rat and mouse gonads was extracted by using the guanidinium isothiocyanate method (16). Poly $(A)^+$ RNA was isolated by oligo dT cellulose (Sigma Chemical Co., St. Louis, MO) extraction. Poly $(A)^+$ RNA samples (35 μg) were electrophoresed in agarose gels containing formaldehyde and transferred to Gene Screen nylon membrane (DuPont, NEN, Boston, MA) by using standard conditions. The FGF-8 specific cDNA (17) was [^{32}P]dCTP labeled by the random oligonucleotide priming method (18) with Ready-to-go labeling kit (Pharmacia, Uppsala, Sweden). Hybridization was carried out in standard conditions and filter was exposed to Kodak X-Omat film at −70°C. Corresponding hybridization of the blot with ^{32}P end-labeled oligo$(dT)_{15}$ (19) was used as a control for poly $(A)^+$ RNA loading. For quantification, the intensities of the FGF-8 mRNA and oligodT bands in autoradiographs were quantitated by densitometry with a Microcomputing Imaging Device M4 (Imaging Research Inc., Canada, 1995) by using the 2.1 Program Version. The relative mRNA levels were calculated using ^{32}P end-labeled oligo$(dT)_{15}$ as a reference.

In situ hybridization. Mouse and rat embryos, and adult ovaries and testes were fixed in 10% buffered formalin at room temperature for 24 h, dehydrated in ethanol, cleared in xylene, and embedded in paraffin. Sections (5 μm) were placed on microscope slides treated with Denhardt's solution (0.02% polyvinylpyrrolidone [Sigma Chemical Co., St. Louis, MO], 0.02% Ficoll [Pharmacia, Uppsala, Sweden], 0.02% BSA [Sigma Chemical Co., St. Louis, MO], and 3-strength SSC). FGF-8 antisense and sense probes were prepared by in vitro transcription of linearized pGEM3Z (Promega Corp., Madison, WI) containing the coding region of FGF-8 cDNA (629 bp) (17). Riboprobes were synthesized in the presence of ^{35}S-UTP (Amersham International, UK) using either SP6 or T7 polymerase (Promega Corp., Madison, WI) for the production of antisense and sense transcripts, respectively. The length of the ^{35}S-labeled transcripts was reduced with alkaline hydrolysis to less than 200 bp. *In situ* hybridization was performed as previously described (20). Autoradiography was performed by dipping the slides into Kodak NTB-3 emulsion (Eastman Kodak Co., Rochester, NY), exposing for 11-54 days in desiccant containing boxes at 4°C, developing in Kodak D-19 developer (Eastman Kodak Co., Rochester, NY), and counterstaining with hematoxylin.

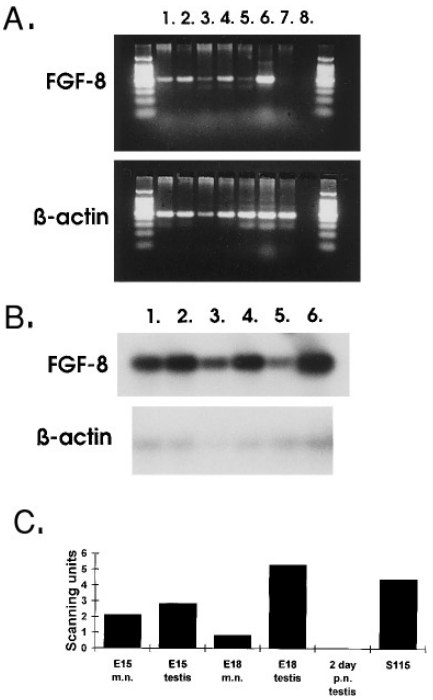

FIG. 2. (A) Detection of FGF-8 mRNA expression in (1) E15 metanefroi (metanefroi from 15-day-old fetuses), (2) E15 testis, (3) E18 metanefroi, (4) E18 testis, (5) testis from 2-day-old postnatal mice, (6) S115 cells (positive control), (7) rat hypophysis (negative tissue control) and (8) H_2O (negative control) by RT-PCR experiments. β-actin RT-PCR experiments were performed on each sample. The amplified fragments were visualized on a 2% ethidium-bromide-stained agarose gel and (B) submitted to Southern blot analysis. (C) Densitometric analysis of Southern blots. The intensity values of FGF-8 were corrected by the corresponding intensity values of β-actin. Relative levels of FGF-8 mRNA [FGF-8/β-actin; scanning units] are shown.

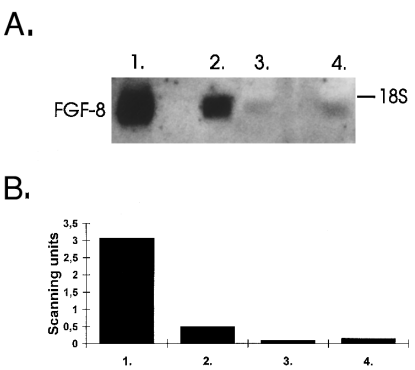

FIG. 1. (A) Northern blot hybridization analysis of FGF-8 mRNA expression in S115 cells (used as a positive control) (lane 1), adult mouse ovary (lane 2), mouse testis (lane 3), and rat testis (lane 4). 2,5 μg of poly $(A)^+$ RNA was loaded on the lane 1 and 35 μg on the lanes 2–4. Exposure time was 10 days. (B) Densitometric analysis of Northern blot. The intensity values of FGF-8 were corrected by the corresponding intensity values obtained after hybridization with oligo$(dT)_{15}$. Relative levels of FGF-8 mRNA [FGF-8/oligo$(dT)_{15}$; scanning units] are shown.

RNA preparation and semiquantitative reverse transcription polymerase chain reaction (RT-PCR). For guanidium isothiocyanate extraction of total RNA (16), eighteen isolated mouse testes freed from the mesonefroi were used from 15-day-old fetuses and sixteen testes from 18-day-old fetuses. From 2-day-old postnatal mice, six testes were pooled for the extraction, respectively. Isolated metanefroi of 15- and 18-day-old fetal mice were used as a positive tissue control. Total RNA was also isolated from rat hypophysis and used as a negative tissue control.

cDNA synthesis was performed at 37°C for 1 hour using 1 μg total RNA with M-MLV reverse transcriptase (Promega Corp. Madison, WI) and oligodT primer (Perkin Elmer Cetus, Norwalk, CT).

FGF-8 and β-actin cDNAs were amplified using either common FGF-8 primers: sense 5′-TTTACACAGCATGTGAGGGAG-3′ and antisense 5′-GTAGTTGAGGAACTCGAAGCG-3′ (8) or β-actin primers: sense 5′-GAAATCGTGCGTGACATTAAGGAG-3′ and antisense 5′-ATACTCCTGCTTGCTGATCCACAT-3′. The cDNAs were amplified by 30 cycles. Each cycle consisted of an incubation period of 1 min 94°C, 1 min 60°C, and 2 min at 72°C. For semi-quantitative

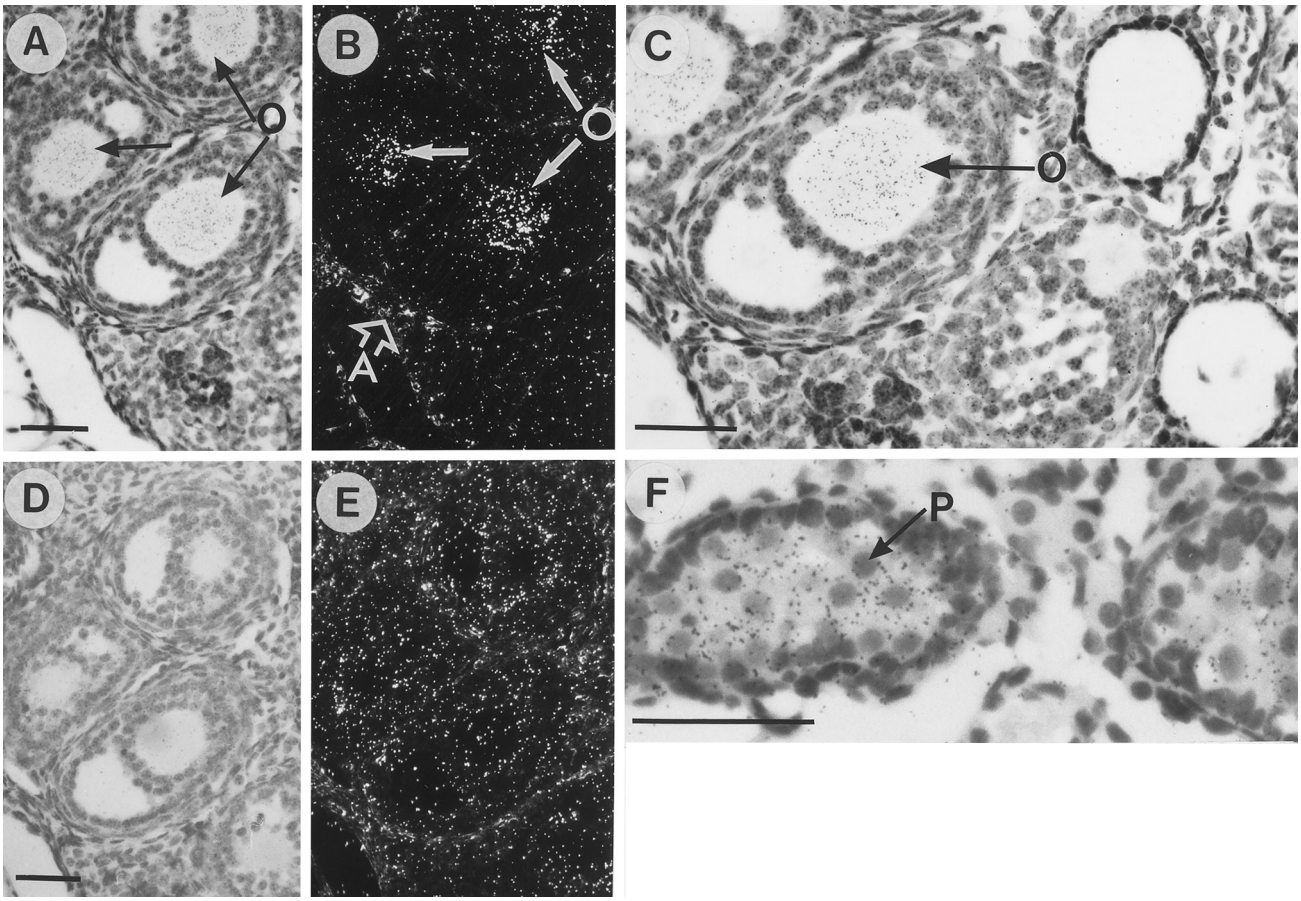

FIG. 3. Localization of FGF-8 mRNA transcripts by *in situ* hybridization in adult mouse ovary and 17-day-old fetal testis. Dark (B) and bright field (A, C) photomicrographs show specific hybridization signal in oocytes (O, arrows) of small and large antral follicles. Autoreflection (A, open arrow) is shown in the dark field photomicrograph (B). No specific labeling was observed in the parallel sections hybridized with FGF-8 sense RNA (D, E). Bright field photomicrograph (F) shows accumulation of specific hybridization signal in 17-day-old fetal prespermatogonia (P). Bar 50 μm.

analysis the PCR products were electrophoresed on a 2% agarose gel and transferred to Gene Screen nylon membrane (DuPont, NEN, Boston, MA) by using standard conditions. Blots were hybridized with [^{32}P] labeled FGF-8 and β-actin probes (Oncor Inc., Gaithersburg, MD) in standard conditions. The intensities of hybridization signals were quantified by densitometry with a Microcomputing Imaging Device M4 (Imaging Research Inc., Canada) by using the 2.1 Program Version.

RESULTS AND DISCUSSION

In the present study the coding region of mouse FGF-8 cDNA (2) was used to study the temporal and spatial expression pattern of FGF-8 mRNA in developing fetal and adult mouse and rat gonads. Northern hybridization of poly $(A)^+$ RNA from adult mouse ovary and testis as well as rat testis with the PCR cloned FGF-8 cDNA recognized an approximately 1.4 kb transcript in each (Fig. 1A) which is consistent with the previous finding (15).

To assign the cells expressing FGF-8 mRNA sections of ovaries and testes were hybridized *in situ* with antisense riboprobe derived from FGF-8 cDNA. In adult mouse ovary, distinct *in situ* hybridization signal for FGF-8 mRNA was specifically localized to the maturing oocytes of growing small and large antral follicles (Fig. 3A-C). The oocytes of the primary follicles, which are metabolically relatively inactive, and atretic follicles showed no FGF-8 mRNA expression. In female fetal gonads, we could not demonstrate FGF-8 mRNA *in situ* hybridization signal in any cell type of fetal 11-19 day mouse or rat 12-19 day ovaries.

In the fetal mouse testis the accumulation of FGF-8 hybridization signal was first detectable in the prespermatogonia of 15-day fetal testis. The expression was at its highest in the fetal 17-day testis (Fig. 3 F) but much lower again in the prenatal 19-day testis. In adult testis, no specific hybridization signal over backgroud was observed. It is noteworthy that despite the large amount of poly $(A)^+$ RNA (35 μg) from adult mouse and rat testes analyzed on the Northern

blot, only a faint hybridization signal from FGF-8 mRNA was obtained. Thus, it is apparent that in adult testis the level of FGF-8 expression is very low. The time course of the expression of FGF-8 mRNA observed in the *in situ* experiments was also confirmed by using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) (Fig. 2). The expression of house keeping gene β-actin was used as a reference.

The timing of FGF-8 expression suggests that it has a specific function in the oocyte maturation, and follicular development as well as in maturation of the seminiferous epithelium of testis. FGF-8 has been found to increase cell proliferation in SC-3 mouse mammary tumor cell cultures (21) and during embryogenesis, ectodermally derived FGF-8 has been suggested to function as a paracrine mitogen that stimulates the onset of limb bud outgrowth (22). Our results show, that the germ cell-specific expression of FGF-8 in rodent gonads coincided with active proliferation of the surrounding somatic cells. The granulosa cells are actively proliferating in the preantral and antral follicles, in which FGF-8 mRNA is expressed but in fetal ovaries the primordial ova, which did not express FGF-8, are surrounded by only a single layer of nonproliferating granulosa cells. Correspondingly in testis, the Sertoli cells, which are the male counterparts of granulosa cells, are mitotically active in the fetal testis (23) at the time when the accumulation of FGF-8 hybridization signal in the prespermatogonia was observed (Fig. 3F). Accordingly, a very low level of FGF-8 expression in adult testis could be expected since the proliferation of murine Sertoli cells decreases markedly after birth and ceases during puberty (24).

FGF-8 function has been shown to be mediated through a variant form of fibroblast growth factor receptor 1 (FGFR-1c) (11). Experiments with the recombinant FGF-8 protein isoforms (a, b and c) have revealed that they interact with different FGFRs (10). Recently, FGF-8b has been shown to activate FGFR2c, FGFR3c and FGFR4 (25). In rat ovary, mRNA for the fibroblast growth factor receptors FGFR-1 (flg) and FGFR-2 (bek) have shown to be expressed in granulosa and theca-interstitial cells but not in oocyte (26). The expression of the FGFRs on the granulosa cells would thus make the paracrine function of the oocyte FGF-8 possible.

Taken together, these results would be consistent with the mitogenic effect of FGF-8 on the somatic components of the germinal epithelia. It is also possible that FGF-8 acts as a survival factor suppressing apoptosis and preventing atresia as previously shown for basic FGF (bFGF) (27), which is expressed in the granulosa cells of the dominant follicles (28). The expression pattern of FGF-8 mRNA in maturing oocytes and fetal prespermatogonia of mouse and rat gonads is shown for the first time in the present study. The developmentally regulated expression suggests, that FGF-8 has a specific paracrine role in the growth and differentation of the somatic cells of the germinal epithelia in both testis and ovary.

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